Isolation of lipid classes from nutritional marine oils: improved extraction strategy and discrimination studies based on the positional distribution of omega-3 polyunsaturated fatty acids on triacylglycerol structures using liquid chromatography tandem mass spectrometry and principal component analysis

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Master degree thesis for the European Master in Quality in Analytical Laboratory

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Contents

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List of Publications

1. Araujo P., Tilahun E., Breivik J.F., Zeng Y. Grung B, A sequential liquid-liquid strategy for extracting triacylglycerols from marine oils and further discrimination studies based on the stereospecific position of the omega-3 fatty acids. (to be submitted to American Journal of Modern Chromatography)

List of Abbreviations

AA	Arachidonic acid
ALA	Alpha linolenic acid
APCI	Atmospheric pressure chemical ionization
CM	Chylomicrons
D/L	Dextrorotary/levorotatory
DAG	Diacylglycerols
DHA	Docosahexaenoic acid (22:6n-3)
DPA	Docosapentaenoic acid (22:5n-3)
ECN	Equivalent carbon number
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid (20:5n-3)
ESI	Electrospray ionization
ETA	Eicosatetraenoic acid
ETrA	Eicosatrienoic acid
FAME	Fatty acid methyl esters
FFA	Free fatty acids
FID	Flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GLC	Gas chromatography liquid chromatography
HPA	Heneicosapentaenoic acid
HPTLC	High performance thin layer chromatography
LA	Linoleic acid
LC-ESI-MS	Liquid chromatography electrospray single mass spectrometry
LC-ESI-MS^2	Liquid chromatography electrospray tandem mass spectrometry
LLE	Liquid -liquid extraction
MAG	Monoacylglycerols
MALDI-TOF-MS	Matrix-assisted laser desorption-ionization time-of-flight
MCFA	Medium chain fatty acid
MCT	Medium chain triacylglycerol
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PL	Phospholipid
PLS	Partial least squares
PUFA	Polyunsaturated fatty acids
RP-HPLC	Reversed phase high performance liquid chromatography
SDA	Stearidonic acid
SIMCA	Soft independent modelling of class analogy
SPE	Solid phase extraction
TAG	Triacylglycerols
TIC	Total ion chromatogram
TLC	Thin layer chromatography

Abstract

The consumer of the 21st century have developed an awareness of the quality of food products in their diet and the impact of these products on their health which in turn has led to an escalating consumer demand for ω-3 fatty acids rich oils, functional food, dietary supplements and pharmaceuticals. The analysis of ω-3 rich oils for detecting the presence of adulterants is generally carried out by using complex, time-consuming techniques and expensive and sophisticated instruments. It is therefore essential to establish simple and reliable analytical methods in order to carry out quality assessment and authentication of nutritional ω-3 fatty acids rich products.

The most critical factors clearly affecting the analysis of lipids in a wide variety of samples are the extraction and isolation steps due to the presence of various lipid classes, which in turn demand the pre-separation of the sample prior to fatty acid methyl ester (FAME) compositional analysis by gas chromatography (GC) or liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS^2).

In this work, a simple, rapid, and cost effective novel liquid-liquid extraction (LLE) method is developed to separate triacylglycerol (TAG) and phospholipids (PL) from marine oils. Krill oil was selected as analytical sample due to its content of TAG and phospholipids.The method consists basically of sequentially adding methanol and hexane to the oil sample, separating and washing the phases with solvents of opposite polarities. The result revealed that it is possible to separate the major TAG and PL constituents from krill oil samples. The high performance thin layer chromatography (HPTLC) chromatograms revealed that TAG and PL were absent from the PL and TAG rich fraction respectively.

The thesis also studies the capability of stereospecific positioning for discriminating marine oils based on their number of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) at *sn*-2 position of TAG structures. The fatty acid distribution on TAG molecules was determined by using LC-ESI-MS^2 and a previously developed algorithm. The results were arranged in increasing number of equivalent carbon number (ECN) and the ω-3 PUFAs at *sn*-2 positions were counted and presented in a histogram. The results revealed that discrimination studies based on the *sn*-2 position are reasonable alternatives for discriminating genuine from processed marine oils.

The final aspect studied in the present thesis is the implementation of a new strategy for discriminating marine oils by using the position of ω-3 PUFAs at *sn*-1, *sn*-2 and *sn*-3 combined with principal component analysis (PCA). The novel strategy demonstrated to be a reliable approach to discriminate not only genuine but also processed and intentionally adulterated oils.

1. Introduction

1.1 Background

1.1.1 Lipids

Lipids play a variety of roles in biological systems. They are essential molecules for cells, the basic unit of all life forms. All cells are spatially defined by their plasma membranes and the lipids are basically the building blocks of plasma membranes. This membrane provides structure for cells and organelles and maintains physiochemical properties, creating an environment inside a cell that is necessary for proteins to function and interact. They also serve as a source of energy as triglycerides in mammalian cells [1].

There is no widely accepted definition of lipids (Greek lipos = fat). A broad definition is that it is a group of naturally occurring compounds, which have a general solubility in organic solvents as hydrocarbons, chloroform, benzene, ethers, and alcohols. They include fatty acids and their derivatives, carotenoids, terpenes, steroids and bile acids. This type of definition can mislead, since many of the substances that are now widely regarded as lipids may be almost as soluble in water as in organic solvents. Nowadays it is accepted that lipids can be defined as fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds [1,2].

Lipids are broadly classified into simple, complex and derived, which are further subdivided into different groups. Simple lipids are compounds that upon hydrolysis yield no more than two types of primary products per mole, also referred to as neutral or non-polar lipids. Simple lipids comprise cholesterol and cholesteryl esters, free fatty acids, glycerides and waxes (esters of fatty acids, usually long chain, with alcohols other than glycerol). Polar or complex lipids are compounds that upon hydrolysis yield three or more primary products per mole. Complex lipids comprise phospholipids, glycolipids, and sphingolipids. Derived and precursor lipids include fatty acids, glycerol, steroids, other alcohols, fatty aldehydes, hydrocarbons, lipid-soluble vitamins and hormones, which are the building block of simple and complex lipids [2,3].

The study of the lipid classes in their natural environment is termed lipidomics. This is new field of study that focuses on pathways and networks of cellular lipids in biological systems and involves the identification and quantification of the thousands of cellular lipid molecular species and their interactions with other lipids, proteins, and other metabolites [3].

1.1.2 Fatty Acids

A fatty acid is a carboxylic acid consisting of a hydrocarbon chain and a terminal carboxyl group, which can appear as free and part of complex lipids. They play a vital role in storage and transportation of energy, and are essential components of all membranes.

Fatty acids can be classified as essential or nonessential. Essential fatty acids are those that our bodies cannot synthesize and must be obtained through nutritional sources. Examples of essential fatty acids are linoleic acid (18:2n-6), alpha-linolenic acid (18:3n-3) and arachidonic acid (20:4n-6). Conversely, nonessential fatty acids are those that can be synthesized by our bodies. Depending on the number of double bonds they may also classified as saturated and unsaturated fatty acids (Fig 1).

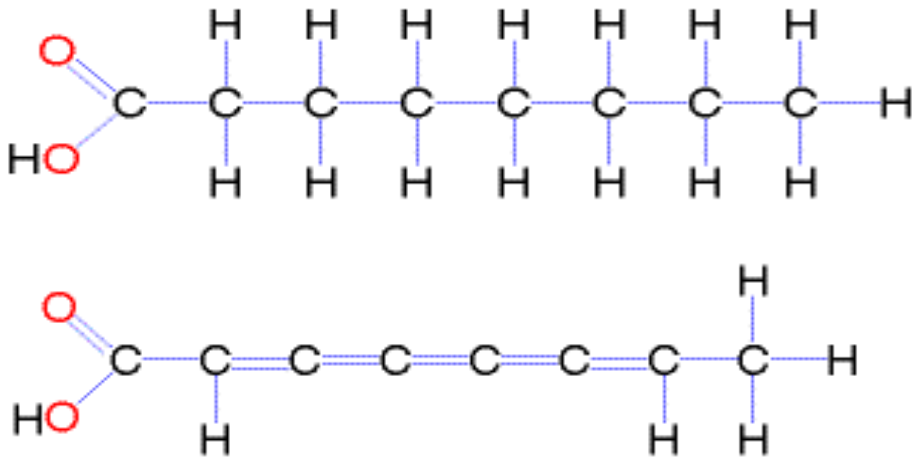

Figure 1 Saturated (top) and unsaturated (bottom) fatty acids

A saturated fatty acid is one in which all the carbons atoms are saturated with hydrogen (Fig 1). Saturated fatty acids have no double bonds and they are generally found in high concentrations in foods such as palm kernel oil, butter oil and coconut oil. The most abundant saturated fatty acid in nature is palmitic acid (16:0). Even carbon numbered fatty acids from 2

- 30 have been found in nature such as arachidic acid (20:0), stearic acid (18:0) and palmitic acid (16:0). Chain length of fatty acids depends on the number of carbons. Saturated fatty acids can be divided into four subclasses base on their chain length: short-chain saturated fatty acids (2-6 carbons), medium-chain saturated fatty acids (8-12 carbons), long-chain saturated fatty acids (14-20 carbons) and very- long-chain saturated fatty acids (21 or more carbons) [4].

The introduction of double bonds in the hydrocarbon chain results in the formation of the unsaturated fatty acids (Fig 1). Unsaturated fatty acids can be categorized into monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). MUFAs and PUFAs can be classified into three sub groups based on chain length. Short-chain unsaturated fatty acids are those with a number of carbon atoms lower than 19 ($C<19$). Long-chain unsaturated fatty acids are characterized for a number of carbon atoms ranging from 20 to 24 and very-long unsaturated chain fatty acids are basically characterized for containing a number of carbon atoms over 25 ($C\geq25$) [4].

The most common MUFA have chain length s between 16 and 22 and only a double bond in cis configuration. The most abundant monoenoic fatty acid in nature is oleic acid (18:1n-9). High concentrations of oleic acid are found in olive oil, canola oil, peanut oil, sunflower oil, safflower oil, and chicken fat.

PUFA are fatty acids with two or more carbon – carbon double bonds. They are found primarily in vegetable oils, nut oils and fish oils. The chemical structure of a long chain PUFA such as eicosapentaenoic acid (EPA, 20:5n-3 or 20:5ω-3) is shown in Fig 2. Two numbers separated by a colon (e.g. 20:5) give the chain length (number of carbon) and number of double bonds respectively. The double-bond position relative to the methyl end is generally indicated by using the notation n-x or ω -x, where x represents the position of the first double bond relative to the methyl end of the carbon chain. For example, the number 20 (in the abbreviated formula of EPA, 20:5n-3) denotes the number of carbon atoms. The second number after the colon (number 5) denotes the number of double bonds with respect to the methyl end, which is numbered as 1. In this way for EPA the first double bond should be found in the carbon number 3 [3,4].

Figure 2 Chemical structure of Eicosapentaenoic acid (EPA, 20:5n-3)

1.1.2.1 Common fatty acids of plant and animal origin

Straight chain fatty acids containing between 16 and 18 carbon atoms and with a number of double bonds ranging from zero to three are common in plant tissue. This type of fatty acids with up to six double bonds are generally separated by methylene groups [1,4]. Systematic names for fatty acids are too long for general use. For example the systematic name 5,8,11,14-eicosatetraenoic acid generally avoided and the shorter alternative arachidonic acid is used instead. Table 1 shows common fatty acids of animal and plant origin with their systematic, trivial and short hand designations.

Table 1 Common fatty acids of animal and plant origin [1]

Systematic Name	**No. of Carbon**	**Trivial Name**	**Shorthand designation**	
			Symbol I	**Symbol II**
Saturated Fatty Acids				
Ethanoic	2	Acetic	2:0	$C_{2:0}$
Butanoic	4	Butyric	4:0	$C_{4:0}$
Hexanoic	6	Caproic	6:0	$C_{6:0}$
Octanoic	8	Caprylic	8:0	$C_{8:0}$
Decanoic	10	Capric	10:0	$C_{10:0}$
Dodecanoic	12	Lauric	12:0	$C_{12:0}$
Tetradecanoic	14	Myristic	14:0	$C_{14:0}$
Hexadecanoic	16	Palmitic	16:0	$C_{16:0}$
Octadecanoic	18	Stearic	18:0	$C_{18:0}$
Eicosanoic	20	Arachidic	20:0	$C_{20:0}$
Docosanoic	22	Behenic	22:0	$C_{22:0}$

Table 1 continued

Systematic Name	No. of Carbon	Trivial Name	Shorthand designation	
			Symbol I	Symbol II
Monoenoic Fatty Acids				
cis-9-hexadecenoic	16	Palmitoleic	16:1n-7	$C_{16:1\ n-7}$
cis-6-octadecenoic	18	Petroselinic	18:1n-12	$C_{18:1\ n-12}$
cis-9-octadecenoic	18	Oleic	18:1n-9	$C_{18:1\ n-9}$
cis-11-octadecenoic	18	cis-vaccenic	18:1n-7	$C_{18:1\ n-7}$
cis-13-docosenoic	22	Crucic	22:1n-9	$C_{22:1\ n-9}$
cis-15-tetracosenoic	24	Nervonic	24:1n-9	$C_{24:1\ n-9}$
Polyunsaturated Fatty Acids				
9,12-octadecadienoic	18	Linoleic	18:2n-6	$C_{18:2\ n-6}$
6,9,12-octadecatrienoic	18	γ- linolenic	18:3n-6	$C_{18:3\ n-6}$
9,12,15-octadecatrienoic	18	α- linolenic	18:3n-3	$C_{18:3\ n-3}$
5,8,11,14-eicosatetraenoic	20	Arachidonic	20:4n-6	$C_{20:4\ n-6}$
5,8,11,14,17-eicosapentaenoic	20	EPA	20:5n-3	$C_{20:5\ n-3}$
4,7,10,13,16,19-docosahexaenoic	22	DHA	22:6n-3	$C_{22:6\ n-3}$

1.1.2.2 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) are considered good fatty acids (especially those from the ω-3 series) due to their numerous health benefits and their role in biochemical processes of animals, including fish and humans. They are crucial for the syntheses of prostaglandins, regulation of inflammatory functions of the body, vasoconstriction and vasodilatation, brain signalling to prevent overeating or weight loss, transport and oxidation of cholesterol, etc. In addition, PUFAs participate in cell membrane formation and can be found in seafood like salmon, seal, halibut, phytoplankton and certain plant oils such as sunflower-seed, corn, sesame and soy oil.

PUFAs can be classified into two major types: omega-6 (ω-6 or n-6) and omega-3 (ω-3 or n-3) fatty acids. The ω-3 and ω-6 PUFAs have received a lot of attention in the scientific community in the last 30 years and are characterized for exhibiting their first double bond three and six carbons away from the methyl end respectively [5].

1.1.3 Omega-3 and omega-6 fatty acids

Certain unsaturated fats are very beneficial and should be purposefully consumed due to their content of ω-3 and ω-6 long-chain (LC) PUFAs. These two typed of PUFAs (ω-3 and ω-6) are essential for health because they cannot be produced by the body and must come from food. ω-3 PUFAs are needed for brain and eye development of the growing fetus during pregnancy, improve blood circulation, reduce the progression of heart diseases and for retaining health throughout life. In addition, ω-3 PUFAs have been shown to play vital roles in inhibiting inflammation and even cancer.

A total of eight ω-3 fatty acids are involved in human nutrition through natural fatty acid biochemistry, cell and tissue structure and function. The fatty acids include alpha-linolenic acid (ALA), stearidonic acid (SDA), eicosatrienoic acid (ETrA), eicosatetraenoic acid (ETA), eicosapentaenoic acid (EPA), heneicosapentaenoic acid (HPA),docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). Among them the most commons are eicosapentaenoic (EPA), docosahexaenoic (DHA), and alpha- linolenic (ALA) acids, see Fig 3a [6-9].

The ω-6 fatty acids, such as linoleic and arachidonic acid, are playing an important role in brain and heart function and mainly found in most of the vegetable oils (e.g. corn, sunflower, safflower, and soy), salad dressing, nuts, whole wheat bread and chicken. The most common form is linoleic acid (LA) accounts for 85 -90% of dietary ω-6 fatty acids see Fig 3b [6-9].

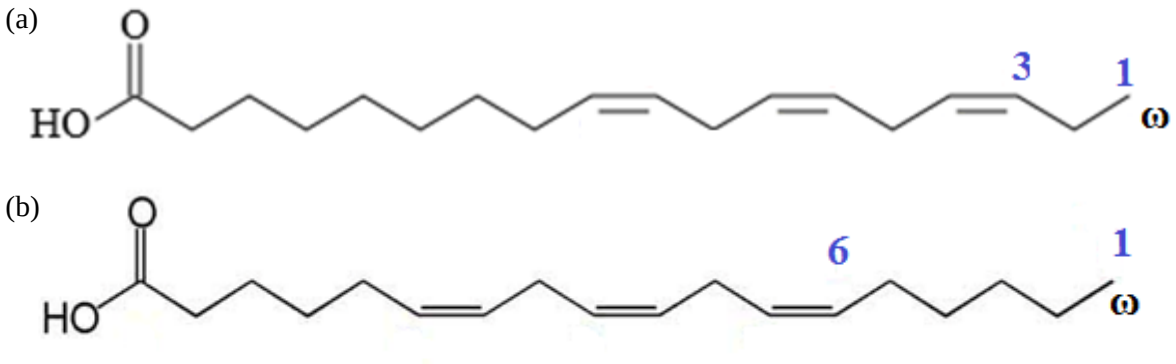

Figure 3 Chemical structure of representative polyunsaturated fatty acids (cis configuration) (a) alpha linoleic acid, ω-3 (18:3n-3 also 18:3ω-3) and (b) gamma linoleic acid, ω-6 (18:3n-6 also 18:3ω-6).

The minimum recommended intakes of ω-3 PUFA is 0.4-0.6 g/day whereas ω-6 PUFAs is 2.4-7.2 g/day. It should be stressed that this is the minimum intake to avoid clinical symptoms of deficiency. It has been suggested that the ratio between ω -3 and ω -6 fatty acids should be 1:4 as compared to 1:20-25 in modern dietary habits. Today we consume 20-25 times more ω-6 fatty acids than ω-3 fatty acids. This imbalance may expose for inflammation and disease such as diabetes, hypercholesterolemia, autoimmune disorders and cancer. Because ω-3 fatty acids are necessary for normal growth, development and metabolism throughout life, they should be abundant in the diets of all humans [6].

1.1.4 Desaturation and elongation

As mentioned above the human body cannot synthesize essential fatty acids (EFAs), however they are important to human health; for this reason, EFAs must be obtained from food. There are two EFAs in human nutrition: α -linolenic acid (ω-3 PUFA) and linoleic acid (ω-6 PUFA), which serve as precursors of other important compounds. For instance, ALA is a precursor for the ω-3 pathway and is metabolized into EPA and DHA. Likewise, LA is the parent fatty acid in the ω-6 pathway and is metabolized into arachidonic acids (AA).

The ω-3 and ω-6 fatty acids are two different groups but they used the same enzymes in some steps through their biosynthesis process. The detailed process involves metabolization by desaturation (extraction of hydrogen) and elongation (addition of carbon atoms) to longer and more unsaturated fatty acids with specific properties as shown in Fig 4 [10].

For example, α-Linolenic acid undergoes desaturation to form stearidonic acid (SDA, 18:4n-3), which by the action of Δ6-desaturase is elongated to eicosatetraenoic acid (ETA, 20:4n-3), which is further desaturated by Δ5-desaturase to eicosapentaenoic acid (EPA, 20:5n-3). Docosahexaenoic acid (DHA, 22:6n-3) is synthesized through the addition of double bond by the Δ4-desaturase to docosapentaenoic acid (DPA, 22:5n-3), which is in turn synthesized from EPA by elongation. The excess of LA can interfere with the metabolism of ALA; this is due to competition between the substrates and due to product inhibition, [11]. Once ω-3 fatty acids have been eaten, the body metabolizes ALA into EPA, DPA and DHA, even though at low efficiency. Approximately 5% of ALA is converted to DHA by the human body [12].

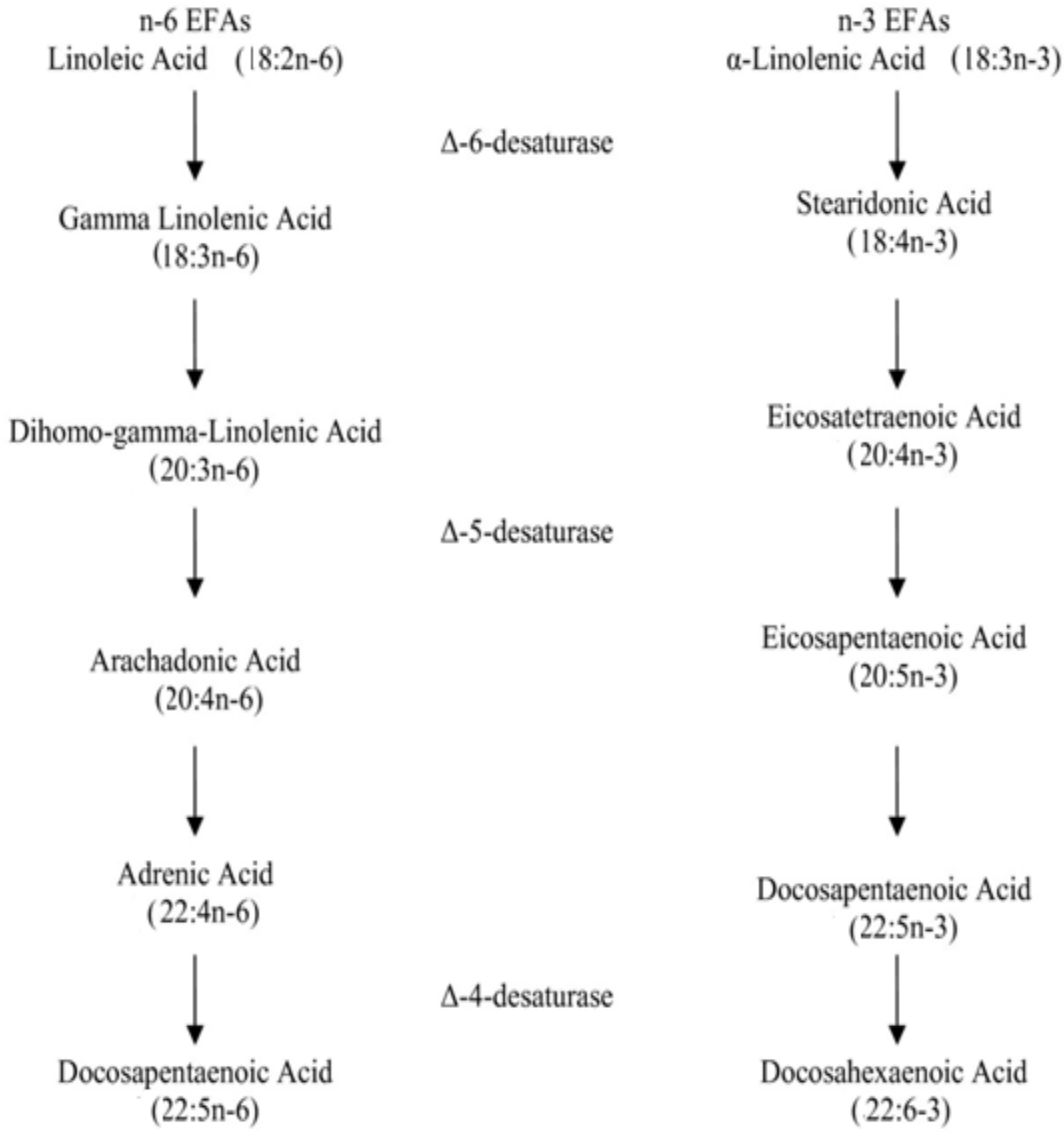

Figure 4 Elongation and desaturation of long chain polyunsaturated fatty acids

1.1.5 Source and composition of marine oils

Lipids from marine sources have gained significance due to the recognition of the rational and health related benefits of PUFAs in prevention or possible curing of human diseases. In most marine organisms lipids are the second largest constituent after protein. Fish like salmon, herring, mackerel and blue whiting contain significant amounts of stored fat in their skeletal muscles and skin while other fish such as cod, halibut and shark have lipids stored mainly in their liver. Marine mammals such as seal, whale and walrus are superbly rich in lipids.

The amount and composition of stored lipids in fish and marine mammals reflect their diet, physiological conditions (like age) and living environment (such as geographical location etc). Triacylglycerols (TAG) are the main components of marine oils while phospholipids (PL) have low contribution to the total amount. Some kind of fish contain (in addition to TAG and PL) wax esters, carotenoids and sterols [13].

1.1.6 Triacylglycerols

One of the main functions of lipids is to serve as a source and storage of energy in mammalian cells as TAGs. Important fats and oils of animal and plant origin contain more than 98% of TAGs, which are classified as simple lipids. This includes all the vegetable oils, such as olive, palm, maize and sunflower oil, and animal fats, such as tallow, lard and butter. The more abundant animal TAGs are milk fats and, their main function is to store energy. Similarly, seed oils serve as a source of energy and structural fatty acids for the developing embryo. TAGs are neutral fats prepared by the combination of glycerol (1,2,3-trihydroxypropane) and 3 fatty acids to form a triester. An example is given in Fig 5 [14].

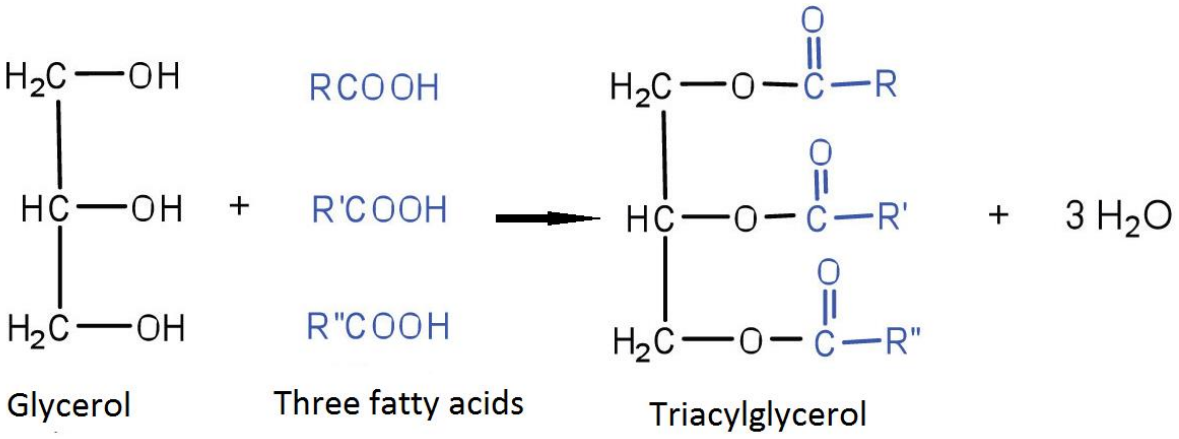

Figure 5 Schematic diagram of triacylglycerol formation

The glycerol molecule itself has a plane of symmetry. However, when the two primary hydroxyl groups become esterified with different acids the resulting glyceride will be asymmetric and become optically active. Simple optically active glycerides can be described without ambiguity by conventional D/L (dextrorotatory/levorotatory) systems but problems arise in application to the complex mixtures of TAGs found in nature. An alternative system of nomenclature, now used by many biochemists dealing with glycerides and its derivatives is described as the "stereospecific numbering" (*sn*-system) and it is recommended by IUPAC-IUB commission [15]. In this system glycerol is stereospecifically numbered (*sn*-glycerol) from top to bottom in the L-form of its Fischer projection (Fig 6) and this numbering is always retained. The two primary hydroxyl groups are positioned at *sn*-1 and *sn*-3 while the secondary hydroxide labeled as position *sn*-2 as shown in Fig 6.

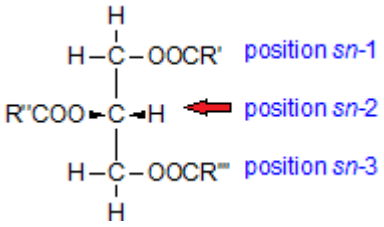

Figure 6 Schematic diagram of triacyglycerol molecule

The three fatty acids at positions *sn*-1, *sn*-2 and *sn*-3 may vary and generate a large diversity of TAGs. The complete hydrolysis of TAGs yields three fatty acids and a glycerol molecule. For example, Fig 7 shows a TAG molecule consisting of three different fatty acids namely palmitic acid, oleic acid and linoleic acid.

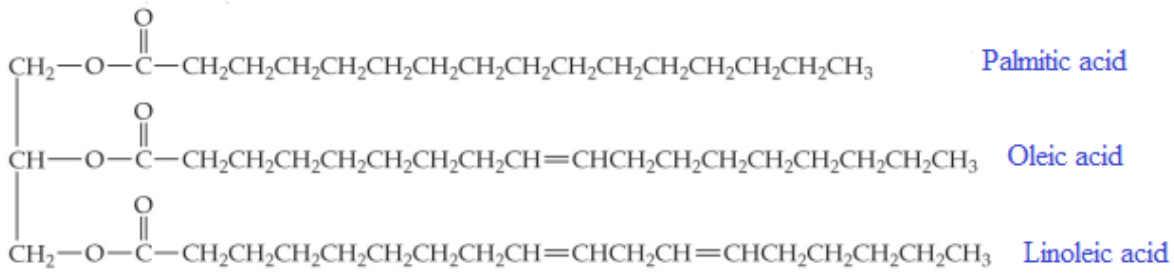

Figure 7 Example of a triacylglycerol molecule

The physical and chemical properties of fats are related to fatty acid composition and distribution in the TAGs. Because of the presence of several different patterns of distribution on the glyceride molecule for a given fatty acid composition, they may produce fats with entirely different properties. The number of possible TAGs increases with increasing the number of fatty acid constituents, for example with three different fatty acid constituents, A, B and C, the number of possible TAGs rises to ten (excluding isomers):A_3, B_3, C_3, A_2B , AB_2, A_2C, AC_2, B_2C, BC_2, ABC; or eighteen if isomers are included [15].

1.1.6.1 Digestion, absorption and metabolism of TAG

An adult consumes approximately 85 g of fat daily, most of it as TAG. The digestion of fats takes place both in the stomach and the intestine, however the major digestion result from pancreatic lipase in the intestine.

The process of fat digestion starts in the stomach by acid-stable gastric or lingual lipases, which is a partial enzymatic hydrolysis into diacylglycerols (DAGs) and free fatty acids (FFAs). Both lipases preferentially hydrolyze the *sn*-3 ester bond resulting in formation of sn-1,2-DAG and the extent is depending on species [17-19]. As most TAG cannot be absorbed into cell walls, transport requires further metabolism of the TAG in the intestine. The degraded product from the stomach enters into intestine (duodenum) stimulates synthesis of the hormone cholecystokinin and causes the gall bladder to release bile acids, which may be released from gall bladder or directly from the liver and act to emulsify the hydrophobic TAGs [19]. In turn, cholecystokinin stimulates the release of the hydrolytic enzyme

pancreatic lipase. The process of hydrolysis is regiospecific so that the pancreatic lipase complex preferentially hydrolyses fatty acid in the *sn*-1 and *sn*-3 position of TAG leaving a 2-monoacylglycerol (2-MAG) that, due to its polar (glycerol) and nonpolar (fatty acid) moieties, functions as an excellent emulsifying agent [20]. On the other hand, pancreatic lipase is relatively inefficient in digesting marine oils and arachidonic acid-containing TAG [21].

The regiospecific structure of dietary TAGs has an effect on the uptake of particular fatty acids and may influence further the lipid metabolism in humans. In humans because of the very low rate of hydrolysis at the *sn*-2 position of glyceride, fatty acids in the *sn*-2 position remain intact as *sn*-2-MAG during digestion and absorption [20]. The lipolysis products including fatty acid, MAGs and DAGs are solubilized together with phospholipids and cholesterol by lysophospholipids and bile salts into micelles and thus absorbed [19-20].
Enzymes involved in the TAG digestion are specific for both stereospecific positions *sn*-1 and *sn*-3, resulting in the formation of a wide variety of MAG, and DAG intermediates. The nature of these intermediates is determined by the positional distribution of the fatty acids in the original TAG molecules.

However, most of these products have specific melting points above body temperature, which may influence subsequent digestion, absorption, and metabolism. FFAs have variable incorporation into mixed micelles, while MAG can readily form mixed micelles and are subsequently absorbed. Unsaturated fatty acids and medium chain fatty acids (MCFAs) are more efficiently absorbed than long-chain saturated fatty acids. The former require lower concentrations of bile salts to achieve emulsification into micellar form, while MCFA can be absorbed in the stomach, after hydrolysis of medium-chain triacylglycerol (MCT) by gastric lipase and can also be solubilized in the aqueous phase of the intestinal contents, where they are absorbed and transported to the liver [19]. However, decreased absorption of long chain saturated fatty acids in the free state may also be due to their high individual melting points above body temperature, which does not tolerate satisfactory incorporation in the liquid phase and the possibility of unesterified fatty acids to form hydrated acid-calcium soaps that are insoluble in aqueous media at the pH of the intestine.[20,22-23].

The FFAs and *sn*-2-MAGs are rapidly taken up by the intestinal cells, via specific carrier molecules but possibly also by passive diffusion, and they are resynthesized into TAGs. The resynthesized TAGs for assembly of chylomicrons (CM) occurs in two pathways, *sn*-2-MAG and phosphatidic acid pathways. The resynthesize of TAGs predominantly through the *sn*-2-MAG pathway, which accounts 80% of the TAG synthesis in fed state while the phosphatidic acid pathway for 20%. The synthesis is stereospecific-favoring reacylation of the *sn*1-position [20]. Chylomicrons are secreted into the lymph and then exported into the plasma in the form of very-low-density lipoproteins. These particles are transported to the peripheral tissues, where they are hydrolysed, releasing FFAs, most of which are absorbed into the adjacent adipocytes and re-utilized for TAG synthesis within the cell [17]. Eventually, the CM remnants are returned to the liver, where the remaining lipids are hydrolysed and absorbed.

1.1.7 Adulteration of dietary oils

The fatty acid composition of the diet affects various aspects of human health. Dietary lipids are essential components of living cells and are incorporated into the lipid structures of cell membranes. They are also important sources for energy and are precursors for numerous biologically active compounds. Dietary oil authenticity is important for a number of reasons including legal compliance, economic reasons, constant quality and health. Authentication is the process of proving that something is true, genuine or valid [24] and in general, food authentication is the process by which food products are demonstrated as complying with their label descriptions. Authenticity may cover adulteration, discrimination characterization, mislabelling, classification and tracing origin.

Deliberate adulteration of pure dietary oils by lower quality oils is an economically profitable practice. These adulterated oils are peril to product authenticity and some time they can be also having a severe consumer health issue. For example, food companies that exploit dietary oils are taking a considerable financial problem if they cannot positively authenticate the purity of oils that they are using in the manufacturing of food products as a result they need a fast, simple, economical and rugged methods that can be used in their laboratory around production area.

1.1.7.1 Authentication of marine oils

Interest on marine oils is increasing due to the health benefits of long-chain ω-3 PUFAs such as docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3). However, differences in price between oils of different origins and qualities, may lead to mislabelling and adulteration [25].

The authenticity of marine oils can be studied by their relative proportion and composition of fatty acids, which is the net result of a wide range of factors, including; diet, season, age, stage of sexual maturity, lipid metabolism and environmental factors [26,27].

A literature review shown that the majority of methods used for authenticity of marine oils are mainly based on stereospecific positioning and fatty acid composition, Table 2.

Fatty acids composition of dietary oils is widely used in the food industry as a marker of purity and adulteration by using gas liquid chromatography (GLC) and comparison of the obtained values with purity criteria [28]. In addition, the fatty acid composition of TAG molecule along with the stereospecific structure of TAGs vary among fish species [29,30] and may therefore be used for authentication of marine oils, since the fatty acid profile of TAGs reflects the diet, species and history of processing [25]. As a consequence of that, TAG patterns and positional distribution of fatty acids usually provide a larger amount of information than a simple fatty acids profiling.

1.1.7.2 Stereospecific authentication of marine oil

Fish oils mainly contain TAG molecules with fatty acids esterified to three stereospecific positions on the glycerol backbone. The positions of these fatty acids are numbered relative to their stereospecific numbering (*sn*) as *sn*-1, *sn*-2 and *sn*-3. The stereospecific distribution of fatty acids in the backbone of TAG is characteristic for different oils and could be used for authentication.The positional analysis of TAGs has traditionally been performed usually based on laborious and time-consuming chromatographic with enzymatic methods [31]. In recent times, various spectroscopic techniques such as matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) [32], reversed phase high

performance liquid chromatography mass spectrometry (RP-HPLC-MS-MS) [33],carbon 13 nuclear magnetic resonance (^{13}C NMR) [34-38], electrospray ionization mass spectrometry (ESI MS) [39] and atmospheric pressure chemical ionization mass spectrometry (APCI-MS) [40] methods have been developed as alternatives to cumbersome and time consuming enzymatic treatments. Nuclear magnetic resonance (NMR) is the most frequently employed technique for authentication of marine oils based on stereospecific positioning as shown in the Table 2, indicating that stereospecific positioning could be used as an alternative approach for authenticity of marine oils in addition to fatty acid profiling.

Table 2 Literature overview of the different analytical techniques used in the authentication of oils derived from fish and marine mammals. The numbers under every technique-column indicate how many times a technique has been implemented for particular oil

Oil	Chromatography				Infrared	Nuclear Magnetic Resonance			Mass Spectrometry	References
	Gas		Liquid							
	GC	GCMS	HPLC APCI-MS	Ag-HPLC APCI-MS	NIR	^{1}H	^{13}C	^{31}P	ESI	
Tuna	1						1			35,**36**
Salmon	3		1				5			35,**37**,**38-41**,42, 48
Cod liver oil	3	1	1		1	1	4	1	1	35,**38** -,40 ,**43** -**45**
Cuttlefish		1							1	39,44
Fish	2		1		1		1			**37**,39,44 ,**45**
Mackerel	1	1					1		1	39,44 ,**45**
Turbot	1									47
Herring			1				1			42,48
Saury		1							1	39,44
Seal	1		1				3			35,**38** -**41**,48

Table 2 Continued

Shark liver oil		1							1	39,44
Whale	2	1							1	35,39,40,44
Crappies	1									49
See bass							1			50
Sardine		1		1					1	39,44 ,50
Anchovy oil	1			1			1			35,**37** ,50
Total	16	7	5	2	2	1	18	1	7	

GCMS = Gas chromatography-mass spectrometry; HPLC = high performance liquid chromatography; APCI-MS = atmospheric pressure chemical ionization-MS; Ag-HPLC= silver-HPLC; NIR= near infrared spectroscopy; ESI = electrospray ionization; APPI= atmospheric pressure photospray ionization; ^{13}C NMR= carbon 13 nuclear magnetic resonance; ^{31}P= Phosphorus 31.

References in bold font are those related to authentication studies by using the stereospecific numbering position of TAGs.

1.1.8 Multivariate analysis and principal component analysis

Multivariate analysis consists of a collection of methods that can be used when several measurements (variables) are made on each individual or object in one or more samples. One aim of multivariate analysis is to decompose mixed data structure into its components. It is applied for a number of different purposes, which are divided into three main groups, i.e., description (explorative data structure modelling), discrimination and classification, and regression and prediction. The commonly employed multivariate statistical techniques include principal component analysis (PCA), partial least squares (PLS) and soft independent modeling of class analogy (SIMCA) [52].

The goal of PCA is to decompose a data table with correlated measurements into a new set of uncorrelated (i.e., orthogonal) variables. It creates new dimensions of data and evaluate a reduced number of independent principal components (PCs) describing the information included in a system of characteristics but partly dependent variables. PC only with certain eigenvalues should be preferably considered and the ranking of the factors is characterised by the amount of variance which they explain [53]. The first PC is the major axis of the points in the p-dimensional space that accounts for maximum amount of variance in the data. The second PC is perpendicular to the first PC and it defines the next largest amount of variation accounts, and so on. Once obtained, the PCs can be graphically plotted in order to search for meaningful distribution patterns that in turn can assist in distinguishing and classifying different set of samples.

2. A simple approach for separating triacylglycerols and phospholipids from marine oil samples

2.1 Background

The study of lipids has gained considerable attention in recent years due to their involvement in many vital biological processes in plants, animals and microorganisms. It is evident that lipid serves as source of energy, carrier of fat soluble vitamins and responsible for maintaining the structural integrity of cells as the principal components of the membranes. Also, lipids are ingredients of all foods and their composition is obviously vital for a good and healthy nutrition.

The major classes of lipids are simple and complex compounds. The former comprises neutral or non-polar substances such as TAGs and the latter comprises polar lipids which are phospholipids, glycolipids, and sphingolipids.

Qualitative and quantitative methods for major classes of lipids are of great importance in research, clinical and quality control applications. One of the most critical factors clearly affecting the analysis of lipids in a wide variety of samples are the extraction and isolation steps. Methods for separating and isolating neutral and polar lipids have been developed in recent years and most of them are based on preparative thin-layer chromatography (TLC) [54-56], solid-phase extraction (SPE) [57-59] and column chromatography [60].

Regardless of its simplicity, preparative TLC is sensitive to sample load, difficult to collect the lipids from the plate quantitatively, generate silica dusts, add trace contaminants such as silica and fluorescent dyes and can be quite expensive in both time and materials and also oxidation of PUFAs often occurs during the process [57]. The popular SPE usually requires considerable amounts of solvent for extraction and clean-up of both neutral and polar lipids [57].Total crude extract of lipids subjected to SPE by using a silica gel stationary phase has been performed with sequential elution of 5 ml of hexane/diethyl-ether (4:1, v/v), 5 ml hexane/diethyl ether (1:1, v/v), 5 ml of methanol and 5ml of chloroform/ methanol/water (3:5:2, v/v/v). The fractions of hexane and diethyl ether, containing neutral lipids, were combined, dried and used for fatty acid analysis after trans methylation. The fractions of methanol and chloroform/methanol/water, containing polar lipids, such as glycolipids and

phospholipids, were combined, dried and used for HPLC/MS and fatty acid methyl ester analysis of the polar fraction. In addition, the fraction of lipid isolated by SPE is very small for quantitative analysis **[**60,61**]**. Column chromatography isolation of lipids requires expensive equipment, copious amounts of solvent, and can be time-consuming [60-62]. Moreover, due to the complexity of lipid extract it is rarely possible to claim that all the lipid classes can be isolated in a single operation. So it is worthwhile to search for methods capable of isolating phospholipid and TAGs.

The most frequently reported analytical method to determine lipids is gas chromatography coupled with flame ionisation detection (FID) [62]. Using GC analysis, lipids are often derivatised to alkyl derivatives in order to increase volatility, improve separation and sensitivity. Moreover, prior to fatty acid profiling by GC it is important to isolate lipid classes, otherwise correlating fatty acid identification with their lipid class becomes difficult [63]. On the other hand, to increase the sensitivity of ultra violet (UV), fluorescent and chemiluminescent detectors a pre- and post-column derivatisation of lipids is usually recommended [64-66].

HPLC analysis of lipids has been suggested as a reliable alternative to GC for accurate quantitative routine analysis [35,40,44,45]. Liquid chromatography mass spectrometry (LC-MS) methods are also very attractive alternatives that could result in a considerable simplification of sample preparation and increasing of sample throughput [66-68].

In particular, LC–MS has been successfully applied to lipid analysis using soft ionisation techniques, such as electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) coupled to single or tandem mass spectrometers [32,39,40,69-72]. However, in LC-MS analysis by using soft ionization, ion suppression is well known and has been demonstrated among lipid classes. For example, the presence of phospholipids can suppress detection of TAG in positive ion electrospray mode (+ESI) but this problem can be eliminated by separation of mixture component of lipids [73,74].

2.2 Objective

One objective of this thesis is to develop a simple and rapid approach to separate triacylglycerol and phospholipids from krill oil samples prior to compositional fatty acid methyl ester (FAME) analysis by GC or positional analysis by LC-MS.

2.3 Experimental

2.3.1 Reagents and standards

Chloroform, diethyl ether, methyl acetate, potassium chloride, copper(I) acetate, ortho-phosphoric acid, isohexane, butylated hydroxytoluene (BHT), acetic acid and hexane used for liquid-liquid extraction (LLE) and high performance thin layer chromatography (HPTLC) were LC grade, from Merck (Darmstadt, Germany). Methanol (HPLC grade, ≥99.9%) for LLE and HPTLC was from Merck (Darmstadt, Germany). Isopropanol used for HPTLC was from Kemetyl (Norway).

The various standards used for HPLTC analysis including PLs (lysoPC, SM, PC, PI, PE), linolenic acid as FFA, trilinolenin, cholesterol, linolenate cholesteryl, methyl linolenate, monolinoleninglyceroland 1,3-dilinoleinglycerol were obtained from Sigma-Aldrich (St. Louis, MO, USA). PS, PA, cardiolipin standards for HPTLC were from Avanti Polar Lipids (Alabaster, Alabama, US). Linoleyl behenate for HPTLC was from Larodan Fine Chemicals (Malmö, Sweden).

Krill oil capsule were obtained from Aker Biomarine (Oslo, Norway). De-ionized water was used throughout the experiment and purified in a Milli-Q system (Millipore, Milford, USA).

2.3.2 Published versus novel Liquid-liquid extraction method

2.3.2.1 Samples treatment using an extraction protocol proposed in the literature

A lipid extraction method based on the use of a single solvent, namely methanol [75] has recently been published. A brief description of this method (MeOH method) is as follows: 0.1 g of krill oil were added into 10 mL of MeOH. After vortex and incubation on ice for 10 min,

the mixture was centrifuged at 10,000 rpm for 5 minutes at room temperature. An aliquot of supernatant was directly submitted to high performance thin layer chromatography (HPTLC) analysis.

2.3.2.2 Optimization of a novel liquid-liquid extraction (LLE) strategy

The novel LLE strategy proposed in the present thesis (Fig 2.1) consists basically of adding sequentially methanol and hexane to the oil sample, separating and washing the phases with solvents of opposite polarities. For example, the methanol phase (rich in PL) is washed with hexane to remove any remaining TAG and the hexane phase (rich in TAG) is washed with methanol to remove any remaining PL. Charcoal is added to the final phases to remove the red color (due to the presence of astaxanthin in the krill oil capsules) and the clean and bright fractions are submitted to HPTLC analysis.

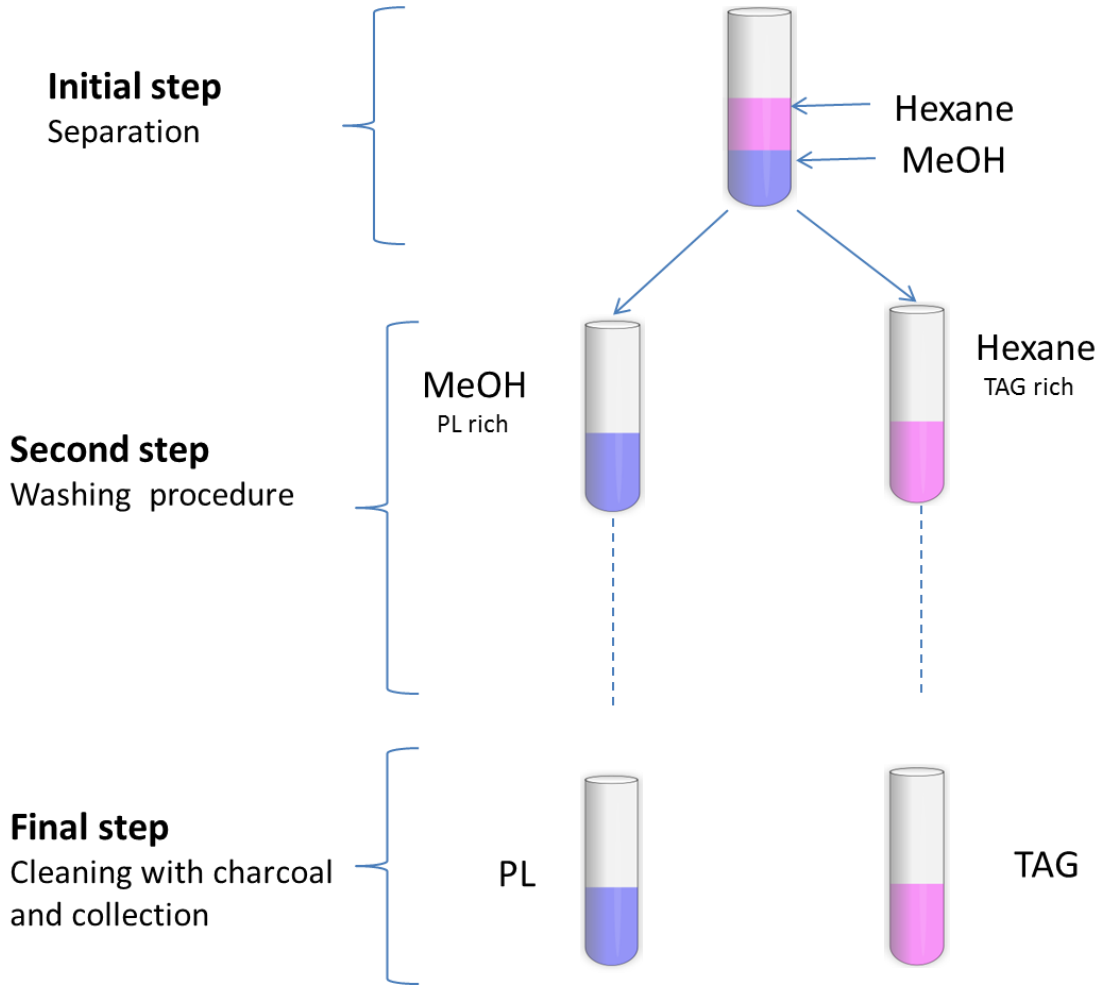

Figure 2.1 Liquid – liquid extraction strategy

To find the best extraction procedure the proposed strategy was optimized, by varying the washing volume of hexane and methanol and the number of washing steps and keeping constant the amount of sample (0.1 g), the initial sample dissolution volume (2 mL hexane and 2 mL methanol) and time of vortex 30 sec. as shown in the Table 2.1.

Table 2.1 Optimization strategy of LLE

Experiment	Washing volume ratio	Washing volume Hexane:Methanol (mL)	No. of washing steps
1	0.5	1:2	2
2	0.5	1:2	3
3	1	1:1	2
4	1	1:1	3
5	1	2:2	2
6	1	2:2	3
7	2	2:1	2
8	2	2:1	3

2.3.3 High performance thin layer chromatography

The HPTLC protocol is part of the methods developed by NIFES and archived as method number MET.NÆR.01-25. 25. Briefly, pure krill oil from a capsule was dissolved in chloroform at 5 mg/mL and sent to HPTLC analysis.The LLE fractions from the hexane and methanol phases were dried under a stream of nitrogen, weighed and redissolved in chloroform at 5 mg/mL and submitted for HPTLC analysis. The various standards used for HPTLC were individually diluted to 0.1 mg/mL by adding chloroform (0.01% BHT). The HPTLC plates 20×10 cm, silica 60 were from Merck (Darmstadt, Germany). The plate was pre-cleaned by eluting the polar solution (KCl: methanol: chloroform: isopropanol: methyl acetate, 9:10:25:25:25, v/v) way up to the top of the plate in a 20×10 cm glass tank. The plate was dried and activated in an oven at 110 ° C for 30 min. Standards(1 µL) and samples (1µL) were applied to the plate with a digital microdispenser (ATS4, Camag, Switzerland). Lipids were first eluted with a polar solution in an automatic development chamber (AMD2, Camag,

Switzerland) until the elution goes up to 48 mm. After 30 min, the plate was wiped and neutral lipids were further eluted with a neutral solution (isohexane:diethyl ether:acetic acid, 80:20:1.5, v/v) up to 88 mm. The plate was dried for 20 min. After removing the plate from the development chamber, it dipped into a glass tank with developing solution (3% copper (I) acetate and 8% ortho-phosphoric acid) and developed for about 10 seconds. The liquid was drained and dried in an oven at 160 °C for 15 min. The Plate was cooled at room temperature and scanned by a D_2 lamp (Scanner3, Camag, Switzerland) at 350nm. The lipid classes in the sample were identified by comparing with the standard band.

2.4 Results and discussion

2.4.1 Optimal LLE strategy

After performing the optimization procedure described in Table 2.1 and represented in Fig 2.1, the best results were those obtained by using experiment number 5 in Table 2.1. The optimal extraction strategy (Fig 2.2) could be summarized in three operational steps as follows:

I. Krill oil (0.1 g) was weighed in a 10 ml pyrex test tube and dissolved in 4 ml of methanol: hexane (1:1, v/v) in the first step. The solution was vortex-mixed for 30 s, centrifuged at 3000 rpm for 1 min and the methanol (MeOH) and hexane (Hx) layers collected separately and labeled as PL rich fraction and TAG rich fraction respectively.

II. An aliquot of 2 mL of hexane is added into the collected PL rich fraction and an aliquoit of 2 mL of methanol is added into the TAG rich fraction. . The solutions were vortex mixed and centrifuged as described previously. After phase separation, the added hexane and methanol solutions were discarded and the initially collected PL and TAG fractions submitted to the second step one more time and the final PL and TAG washed fraction submitted to the third step.

III. Approximately 40 and 15 mg of reactive charcoal were added to the final PL and TAG fractions respectively to remove the red color (due to the presence of astaxanthin in the krill oil capsules). The final fractions were vortex-mixed for 20 s, centrifuged at 3000 rpm for 2 min and the clean and bright supernatants collected, dried under a stream of nitrogen, redissolved in chloroform and submitted to HPTLC analysis.

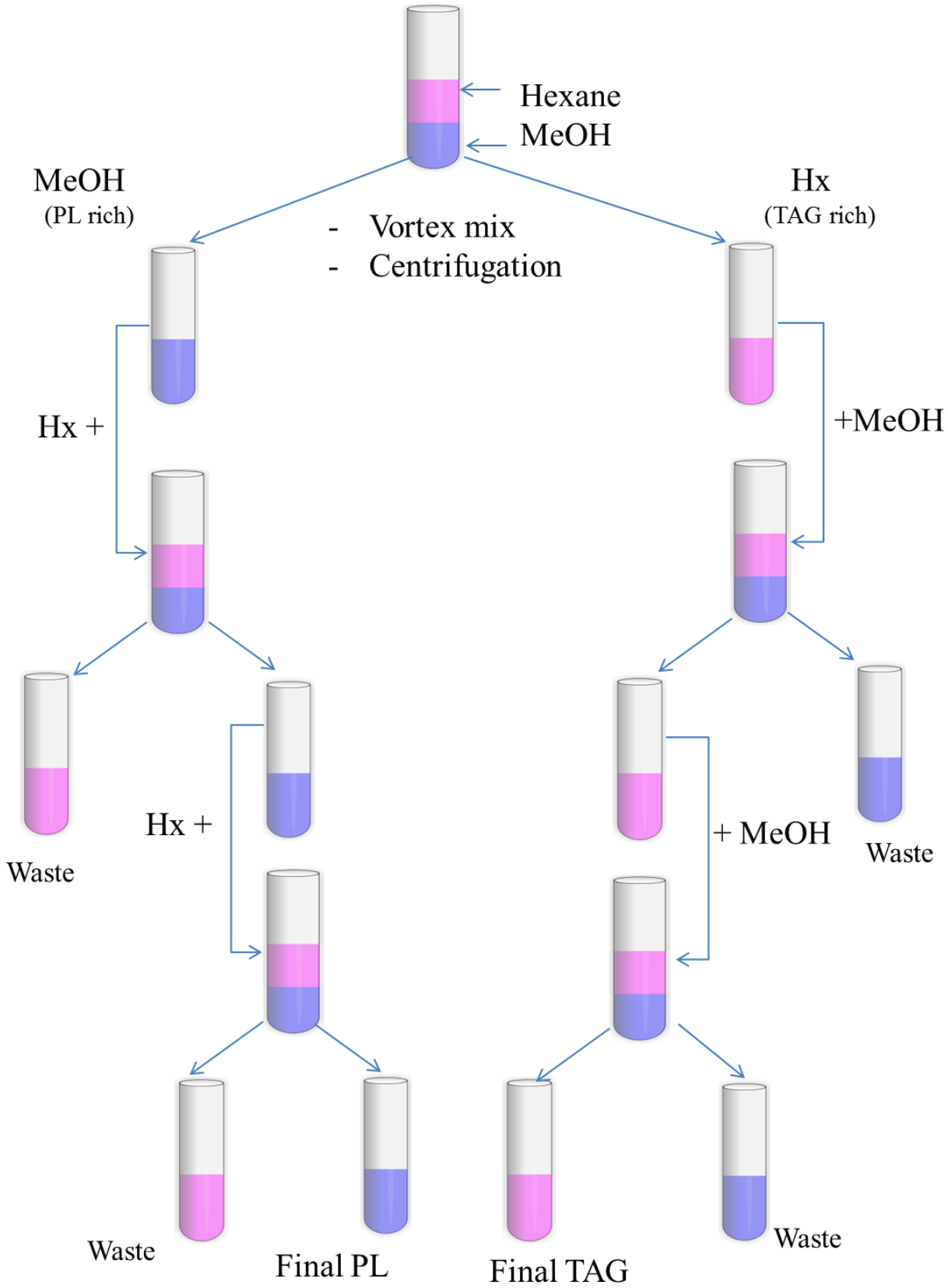

Figure 2.2 Optimal liquid – liquid extraction protocol. Hx= hexane; MeOH= methanol; TAG= triacylglycerol; PL= phospholipids

2.4.2 Comparison of published and novel LLE protocol

Many different lipid extraction methods have been developed in the past decades and most of them are based on the original method developed by Blight and Dyer [76] where three solvents (MeOH, chloroform, water) are used in connection with a 3-step solvent extraction: i) methanol plus chloroform, ii) chloroform plus water are added to the sample and after phase separation lipids are determined in iii) the chloroform phase.

The novel LLE strategy was compared with an already published extraction protocol [75] by using krill oil commercial capsules. The krill oil was selected as an ideal sample because it contains both classes of lipids (TAG and PL) as shown in Fig 2.3.

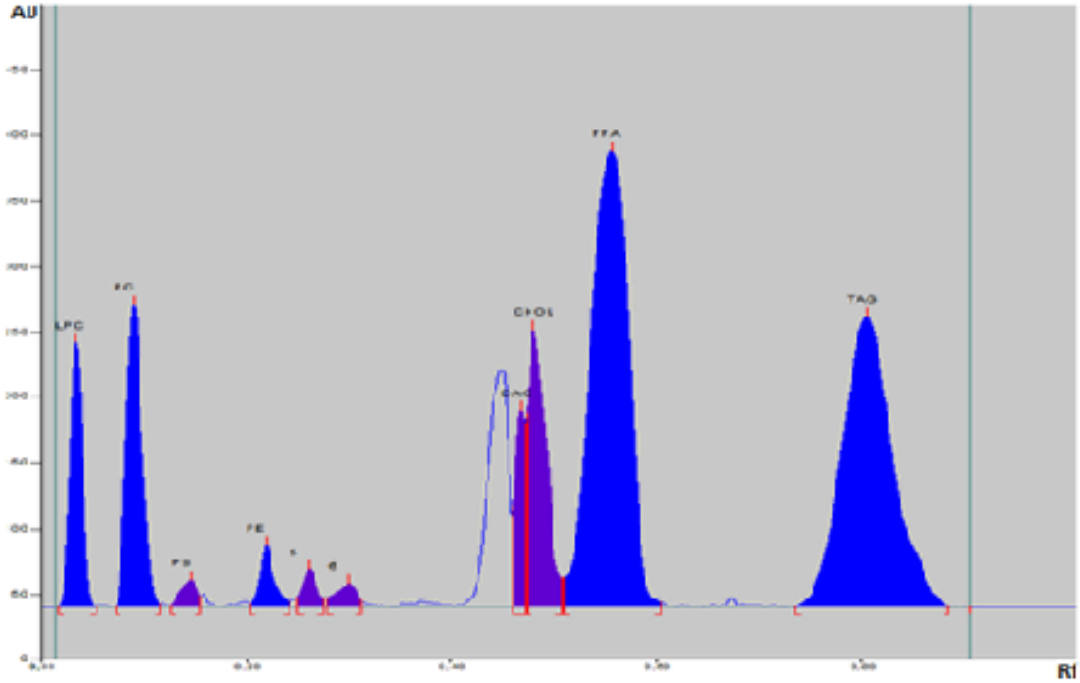

Figure 2.3 HPTLC chromatogram of pure krill oil

2.4.2.1 Published protocol

The PL and TAG content in krill oil were initially separated by using a published method. The selection of this particular method was based on its simplicity. It consists of three simple steps: addition of methanol, centrifugation and collection. The results show in Fig 2.4 revealed that the separation of PL from TAG was not achieved by using this very simple

approach. The HPTLC chromatogram revealed the presence of 8 major components, specifically: lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidyl ethanol amine (PE), phosphatidylserine (PS), diacyleglycerol (DAG), cholesterol, FFA and TAG.

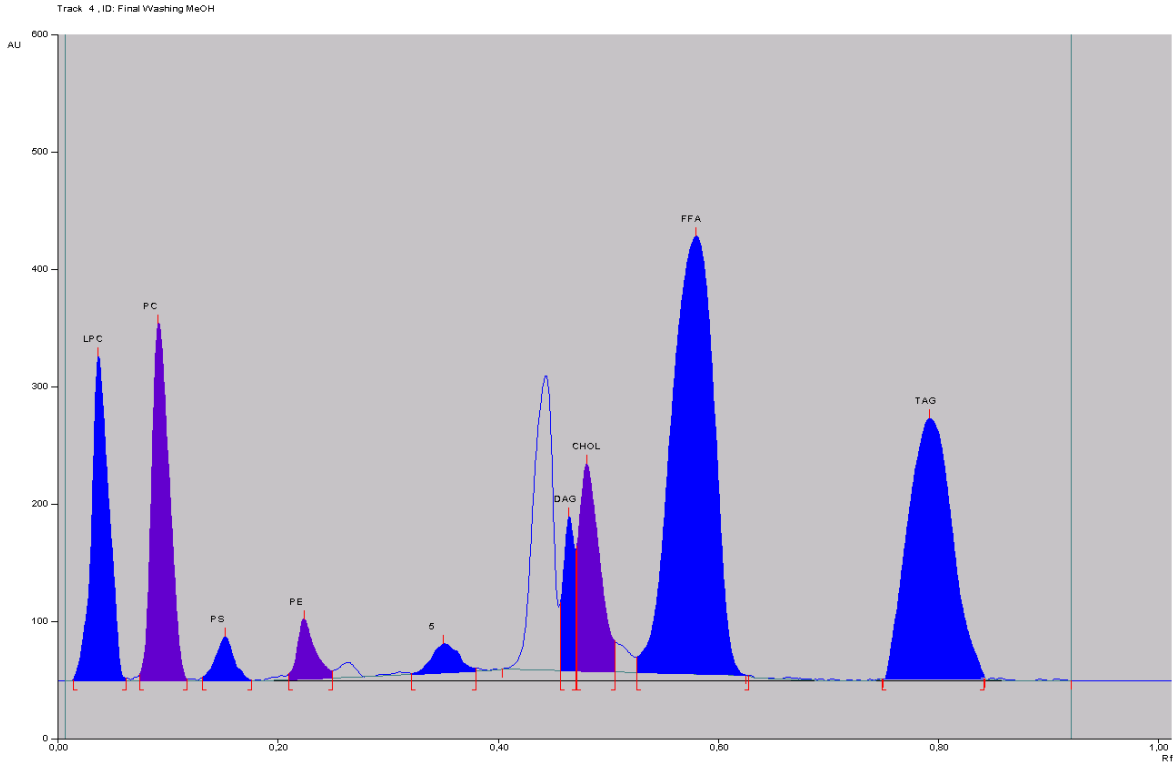

Figure 2.4 HPTLC of krill by using a published protocol (only methanol is used) [75]

2.4.2.2 Novel LLE strategy

The proposed approach, which consists of adding methanol and hexane to isolate PL from TAG and subsequent washing with hexane and methanol to remove any remaining TAG or PL respectively from the initial fractions, revealed that it is possible to separate the major PL and TAG constituents from krill oil samples (Fig.2.5a-b). The HPTLC chromatograms revealed that TAG and PL were basically absent from the PL and TAG rich fraction respectively. Consequently, both fractions could be submitted confidently for FAME analysis and also for determining the positional distribution of fatty acids on PL and TAG structures.

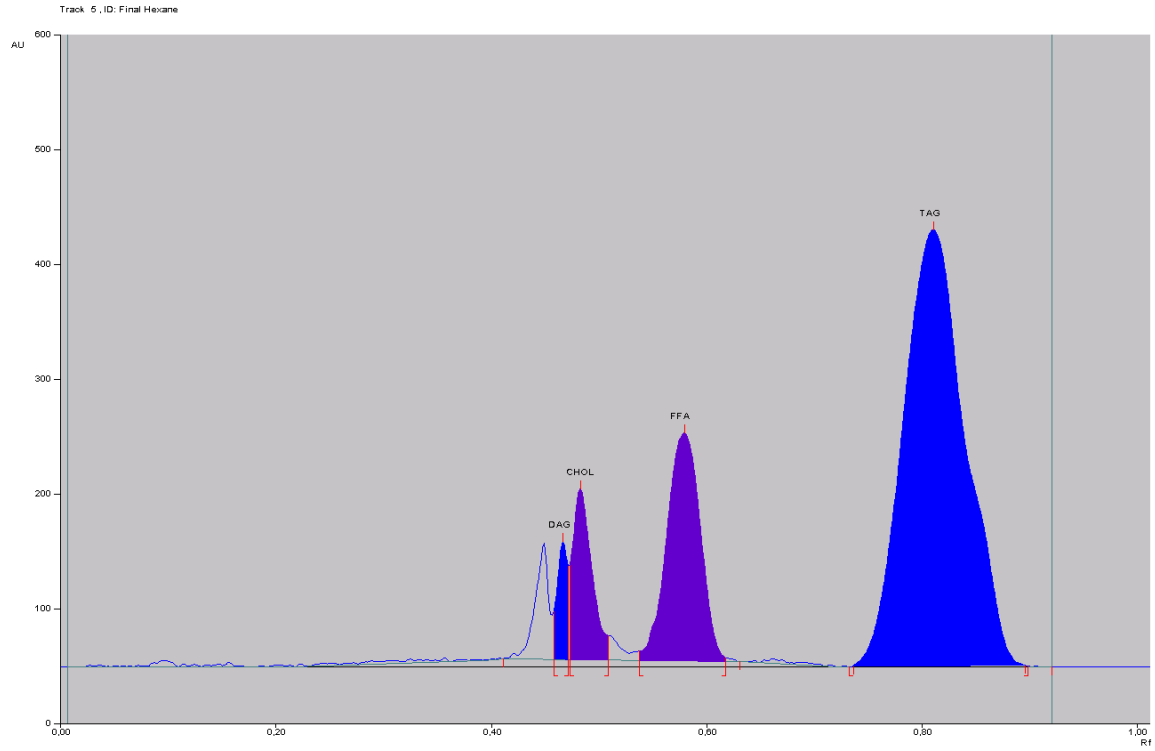

Figure 2.5a HPTLC of final triacylglycerol fraction using the optimal LLE protocol

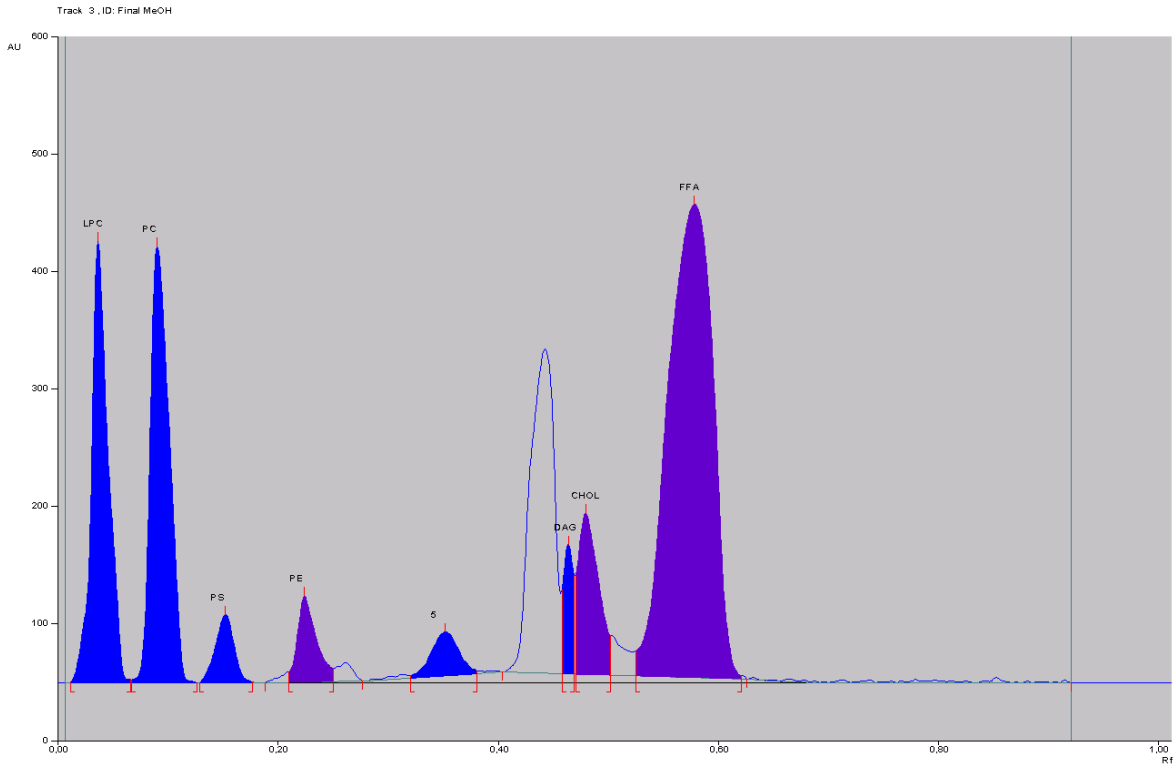

Figure 2.5b HPTLC of final phospholipid fraction using the optimal LLE protocol

It should be highlighted that the methylation method used at NIFES is applicable to PL and TAG and for that reason it is important to separate them in advance to determine the exact contribution of both lipid classes by GC-FAME profiles [77]. The methylation of FFAs is achieved by different procedures to those used currently at NIFES for TAG and PL and consequently it is not expected that they could have an impact in the final GC-FAME profiles of PL or TAG [78].

One important feature of the novel LLE strategy is that it does not require the additions of acid or alkali substances. In addition, the solvents used by the novel strategy are compatible with ESI-MS analysis. Thus, the PL fraction isolated in methanol could be directly used for positional analysis by LC-MS/MS.

It is advisable to dry the TAG fraction in hexane and redissolve it in another solvent (e.g chloroform) prior to any LC-MS/MS. It has been reported that in no circumstances should hexane be employed as the injection solvent. It is so similar in its properties to the stationary phase that it competes with this for the solute molecules, causing peak broadening and it can even cause single components to emerge as double peaks. Unfortunately, hexane is used in so many other chromatographic systems as the injection solvent that novices to HPLC tend to use it in reversed-phase analyses without thinking [79].

Table 2.2 Relative area percentage of lipid classes in hexane and methanol phase

Final washed fraction (%)	**LPC**	**PC**	**PS**	**PE**	**Unknown**	**DAG**	**CHOL**	**FFA**	**TAG**
Hexane	0	0	0	0	0	3	8	18	71
Methanol	16	18	3	4	3	3	7	46	0

The relative area percentages of different PL and TAG shown in the Table 2.2 indicated that in the final hexane phase, TAG accounts 71% and FFA 18% of the total recorded area. While in final methanol phase, PL represents 41% in which PC and LPC accounts 34% of the total area. By considering the relative area percentage of PL and TAG in the final fractions and wastes; it can be therefore, concluded that the extraction yields were approximately 86% of PL and 96% of TAG.

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In some samples, the PL content is relatively low compared to the neutral lipids for such case, prior isolation of PL fraction is needed in order to increase sensitivity of the instrumental technique, such as HPLC analysis. In addition, the presence of phospholipids can suppress detection of TAG species in positive ion electrospray mode (+ESI). Therefore the application of the new developed sequential LLE method could help to alleviate all these problems.

2.5 Conclusions

This new approach is simple, rapid and cost effective for the extraction of major classes of lipids from marine oils with high efficiency and without requiring special equipment. It can be easily applied in different laboratories like research institutes and universities. The most attractive feature of the new LLE protocol in addition to its simplicity is that it helps saving energy, reduces gas emission and creates healthier lab environment and lessen its impact on the environment as a whole when compared with traditional and sophisticated preparative liquid chromatography instrument

.

3. Discrimination of seal oil and cod liver oil based on the *sn*-2 stereo specific position of omega-3 polyunsaturated fatty acids on the backbone of triacylglycerol molecules

3.1 Background

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are necessary for human health and can be found in fish (e.g salmon, tuna, cod fish, halibut), other seafood including algae and krill, some plants and nut oils. The ω-3 PUFAs are considered essential fatty acids and for those people unable or reluctant to boost dietary fatty fish intake, marine oil ω-3 dietary supplements are available like seal oil and cod liver supplement. For example, seal oil supplements primarily from harp seal blubber are manufactured in Canada and contain high levels of DPA and balanced levels of DHA and EPA and it offers privileged properties, such as easier digestibility and better assimilation than other ω-3 sources [80-82].

Interest in seal oil as a source of long chain ω-3 fatty acid began some forty years ago, when it was reported, in the mid-70th, that Greenland Eskimos exhibited low risk of heart disease and cancer in comparison to Western populations. The observed difference in the two populations was immediately attributed to the Eskimo diet which consists mostly of fats of marine mammalian origin like seal oil and whale [83, 84].

The ω-3 fatty acids in seal oil are mainly located in *sn*-1 and *sn*-3 position of triacylglycerols (TAGs), while in fish oil the ω-3 fatty acids are basically located in the *sn*-2 position of TAGs. The difference in the positioning of the ω-3 fatty acids on TAGs might influence the uptake and bioavailability of ω-3 fatty acids due to the stereospecificity of the pancreatic lipase in the gastrointestinal tract for breaking positions *sn*-1 and *sn*-3 leaving position *sn*-2 intact, which is the main reason for the superior effect of seal oil compared to fish oil in disease prevention and potential health benefits [85].

Authentication of dietary marine oil supplements can be accomplished through compositional analyses of the lipids present in such oils [86]. It is known that natural marine oils harvested from fish and seals are typically composed of TAG in which three fatty acids are esterified to

glycerol. Recently, the use of RP-HPLC/APCI-MS with statistical models for the analysis of seal oil and forensic identification was explored [48].

The potential adulterants of seal oil are cod liver and seed oils [87]. Due to the plausible advantage of seal over fish oil as ω-3 supplement, which include enhanced resistance to oxidation and favorable ω-3 composition and position, which resultant in health benefit [88]. Finding methods to check adulteration or distinguishing marine oil harvested from fish and seal is vital in terms of marketing and health benefit.

The analysis of ω-3 rich oils for detecting the presence of adulterants is generally carry out by using complex, time-consuming and tedious chemical/enzymatic hydrolysis methods such as Grignard reagent or lipases analyses [89-91]. Lately, the use of sophisticated high resolution nuclear magnetic resonance spectrometry methods (^{13}C-NMR or ^{1}H-NMR) have been introduced for the stereospecific analysis of TAG molecules in ω-3 rich oils [34-38].

If low amounts of ω-3 PUFAs located at position *sn*-2 of TAG molecules are expected in a regiospecific analysis of seal oil, and high amount of ω-3 PUFAs are expected in the same regiospecific position for fish oil in general, then it is possible to postulate that this particular stereospecific position (*sn*-2) is the most relevant to discriminate between these particular nutritional oils (seal oil and cod liver oil). The positional distribution of ω-3 PUFAs located at position *sn*-2 of TAGs can be carried out by using an already published LC-ESI-MS^2 strategy and an automated TAG prediction algorithm, which enables identifying the relative arrangement of the acyl groups on the glycerol backbone of dietary oils [92].

3.2 Objectives

- To discriminate different kinds of nutritional oils by using their FAME GC profiles
- To discriminate seal oil and cod liver oil based on the stereospecific positioning of particular ω-3 PUFAs on TAG structures, specifically EPA, DHA, 18:4n-3 and 16:4n-3 at *sn*-2 position using LC-ESI-MS^2.

3. 3 Experimental

3.3.1 Reagents and samples

Methanol (HPLC grade, ≥99.9 %), acetonitrile (LC grade, ≥99.8 %), ammonium acetate (mass spectrometry grade, 99%) and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hexane, LC grade was from Merck (Darmstadt, Germany). Isopropanol used for HPLC was from Kemetyl (Norway). NaOH, BF_3 in methanol (20 % w/v) and chloroform were purchased from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT) and BF_3 in methanol (14 %) were purchased from Sigma-Aldrich Co. USA. FAME standards were purchased from Nu-Chek Prep (Elysian, MN), the nonadecanoic acid methyl ester (C19:0) internal standard was from Fluka (Buchs, Switzerland). De-ionized water was used throughout the experiment and purified in a Milli-Q system (Millipore, Milford, USA). Cod liver oils were from Peter Möller, Lysaker and Axellus AS,Oslo, Norway. The salmon oil was from Havnegater, Sortland, Norway. Two different batches of harp seal oil (*Phagophilus groenlandicus*) were from Rieber Skinn A/S, Bergen, Norway and the other seal oil was from JFM Sunile A/S, Os, Norway. The commercial ω-3 supplements obtained from a local pharmacy were Natur-Omega Naturhuset AS, Vøyenenga, Norway. Krill oil capsule was obtained from Aker Biomarine Oslo, Norway. The fish and fish plus evening primrose oil (Omega Woman) were from Nordic Natural AS, CA,USA. The herring, blue whiting and sand eel were kindly donated by Veronika Sele from NIFES.

3.3.2 Gas chromatography

3.3.2.1 Fatty Acid Methyl Esters (FAME) protocol

A 50 µl (50 mg) of sample are mixed with 2 ml BF_3/CH_3OH and 5 mg (1 mg/10 mg) of C19:0 internal standard. The mixture is heated at 100 °C for 1 h and cooled down to room temperature. Aliquots of 1 ml of hexane and 2 ml of H_2O are added, vortex-mixed for 15 seconds, placed in a centrifuge at 3000 rpm for 2 min and the methyl esters are then extracted from the upper hexane phase. Sample were concentrated depend on the fat content under nitrogen and subjected to GC analysis. This preparation protocol has been published elsewhere [77].

3.3.2.2 GC instrumentation

Analysis of the FAME was performed on a Perkin-Elmer AutoSystem XL gas chromatograph (Perkin-Elmer, Norwalk, Connecticut) equipped with a liquid autosampler and a flame ionisation detector. The FAME samples were analysed on a CP-Sil 88 capillary column (50 m × 0.32 mm I.D. 0.2 μm film thickness, Varian, Courtaboeuf, France). Data collection was performed by the Perkin-Elmer TotalChrom Data System software version 6.3. The temperature program was as follows: the oven temperature was held at 60 °C for 1 min, ramped to 160 °C at 25 °C /min, held at 160 °C for 28 min, ramped to 190 °C at 25 °C /min, held at 190 °C for 17 min, ramped to 220 °C at 25 °C /min and finally held at 220 °C for 10 min. Direct on-column injection was used. The injector port temperature was ramped instantaneously from 50 to 250 °C and the detector temperature was 250 °C. The carrier gas was ultra-pure helium at a pressure of 82 KPa. The analysis time was 60 min. The FAME peaks were identified by comparison of their retention times with the retention times of highly purified FAME standards.

3.3.3 Liquid chromatography

3.3.3.1 Sample preparation

An aliquot of 2 ml of each oil was taken and submitted to the optimized LLE method as described in Chapter 2. The final hexane fraction was dried under stream of nitrogen at room temperature. The dried residues of samples were dissolved into 0.5 ml of acetonitrile: acetone (2:1, v/v) and vortex-mixed for 30s. Every sample was prepared in duplicate and the final products were then individually sent to LC-ESI-MS^2 analysis.

3.3.3.2 LC ion-trap mass spectrometry instrumentation

The TAG analysis were carried out by using an Agilent 1100 series LC/MSD trap, SL model with an electrospray interface, a quaternary pump, degasser, autosampler, thermostatted column compartment, variable-wavelength UV detector and 10 μl injection volume. The Zorbax Eclipse-C8 RP 150 × 4.6 mm, 5 μm (Agilent Technologies, Palo Alto, CA) was kept

in the column compartment at 40 °C and the solvent system in gradient mode consisted of acetonitrile (A), acetone (B) and 10 mM isopropanol:ammonium acetate 90:10 v/v (C) at a flow rate of 0.2 ml/min and UV detection at 254 nm. The following gradient programs was used: at initial 5 min condition 90 % C and 10 % B that was ramped in 5 min to 65 % C,5 % B plus 30% of A and returned to the initial condition in 15 min and subsequently ramped in 5 min to 65 % C,5 % B plus 30% of A and returned to the initial condition in 30 min where it was held for 30 min. Nitrogen was used as nebulizing (50 psi) and drying gas (8 L/min) at 350 °C. The ESI source was operated in positive ion mode and the ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option. Data acquisition and processing were controlled by MSD trap control version 5.2 from Agilent.

3.4 Principal Component Analysis

Principal component analysis (PCA) is used to reduce the dimensionality of multivariate data. It is an appropriate way to reduce data sets containing high numbers of variables. By reducing the number of original variables to a smaller number of independent variables, this approach highlights fundamental differences between groups of objects and variables. Some of the articles reported in Table 2 used multivariate data analysis, which has been used in marine oil discrimination. Two-dimensional PCA score plots were created on the normalized data in order to reduce the number of variables. The PCA score plot of the FAME profiles from the various oils was computed with the software package Statistica version 12 (Statsoft. Inc.1984-2014).

3.5 Results and discussion

3.5.1 GC analysis

All oils were analyzed in triplicate and lipid profiles of the various injected oil samples, expressed as mg-FAME/g-sample were arranged in a data matrix consisting of 27 rows, representing the triplicates of various analyzed oils and 34 columns representing the individual FAME detected by GC.

The 27 × 34 matrix was submitted to PCA for data exploration and a total of five PCs grouped in decreasing order of variance extracted. The total FAME profiles were used to discriminate the different oils, with especial emphasis on seal oil and cod liver oil, which are essential for the second objective of the present study.

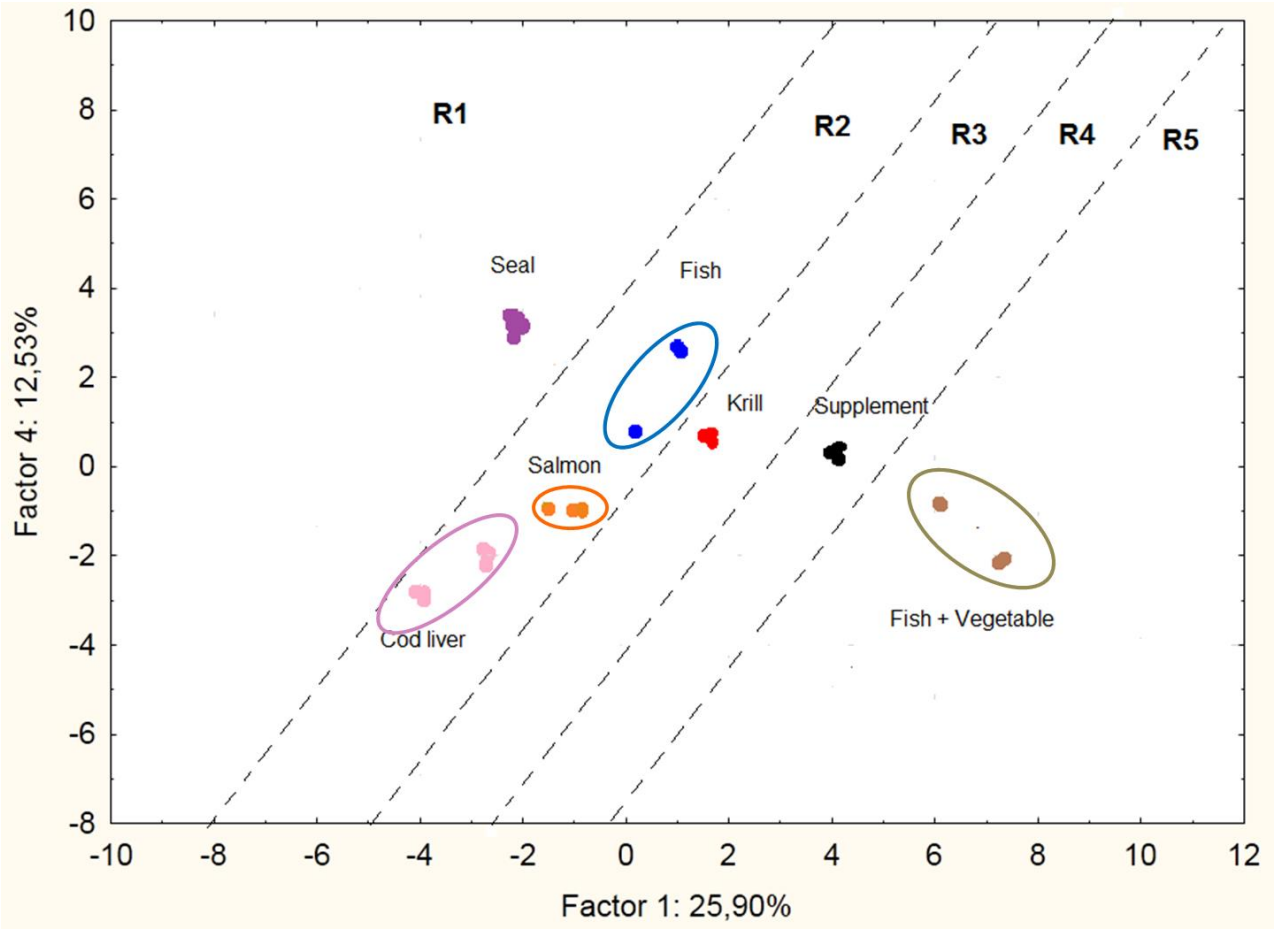

Figure 3.1 PC1 and PC4 score plot for the different kind of oils obtained by using the full FAME profiles

A plot of the scores of PC1 and PC4 explaining 38.43 % demonstrated that it is possible to differentiate five main regions designated as R1 to R5 in Fig 3.1. The regions in questions differentiate seal (R1), fish (R2), krill (R3), supplements (R4) and fish+vegetable (R5). PC1 discriminates between animal, supplements and mixture of animal+vegetable. It must be said that the capability of GC and PCA to discriminate different kind of animal, plant and supplement oil has been demonstrated previously [40].

3.5.2 LC-ESI- MS^2 Analysis data

After confirming the discrimination by GC, the LC-MS^2 data was used to determine the discrimination capability based on the positional distribution of ω-3 fatty acids on the *sn*-2 position of TAGs. The TIC+MS^2 data was exported to netCDF file by Data Analysis for LC/MSD Trap Version 3.3 (Bruker Daltonik GmbH Inc., Billerica, MA, USA). The netCDF file was then exported to a Matlab file and submitted to the developed automated TAG prediction algorithm for identification of TAG species in oil samples. The results were arranged in increasing number of effective carbon number (ECN) and all ω-3 PUFAs at *sn*-2 positions were counted and the average of replicate measurements was used to generate the corresponding histograms.

3.5.2.1 Discrimination of seal and cod liver based on *sn*-2 position

To inspect the distribution pattern of PUFAs in the *sn*-2 position for seal and cod liver oils, the number of PUFAs were plotted against their ECN as shown in Figs 3.2 and 3.3. The histogram of seal oil (Fig 3.2) indicates as expected that seal oil contain low levels of ω-3 PUFAs in *sn*-2 position, whereas cod liver (Fig 3.3) exhibited a higher number of ω-3 PUFAs at the same stereospecific position (*sn*-2), which was approximately three times that of seal oil. This observation agreed with the reports on regiospecific location of ω-3 PUFAs on TAG backbone [85].

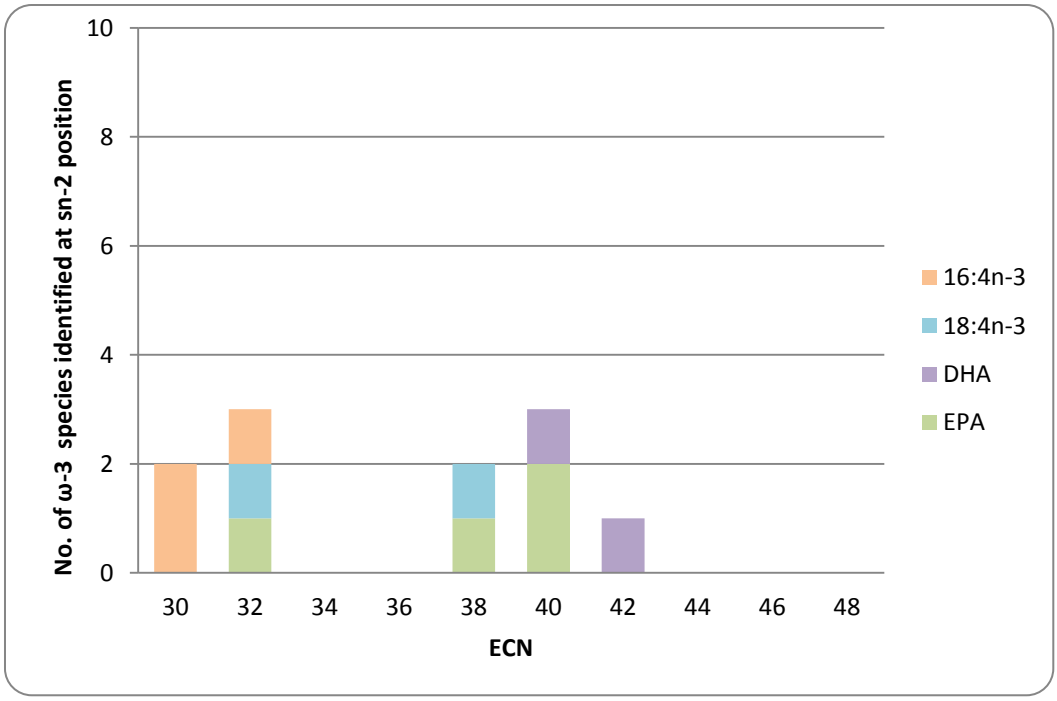

Figure 3.2 Number of ω-3 PUFAs at *sn*-2 position versus equivalent carbon number (ECN) for seal oil

The number of ω-3 PUFAs in cod liver were distributed in a wider ECN and observed even distribution in the range of 36-48 ECN, while in seal oil absence of PUFAs at ECN 34 and 36 were noted. In contrast, cod liver at ECN 34 contains a maximum number of PUFAs, which could be one of the discriminating points in addition to ECN 44-48. Moreover, ECN 32 in seal oil contains three ω-3 PUFAs, which was not seen in other ECN within seal oil, whilst in cod liver oil as noted in Fig 3.3 ECN 32 is completely absent which, could be a source of differentiation between seal and cod liver.

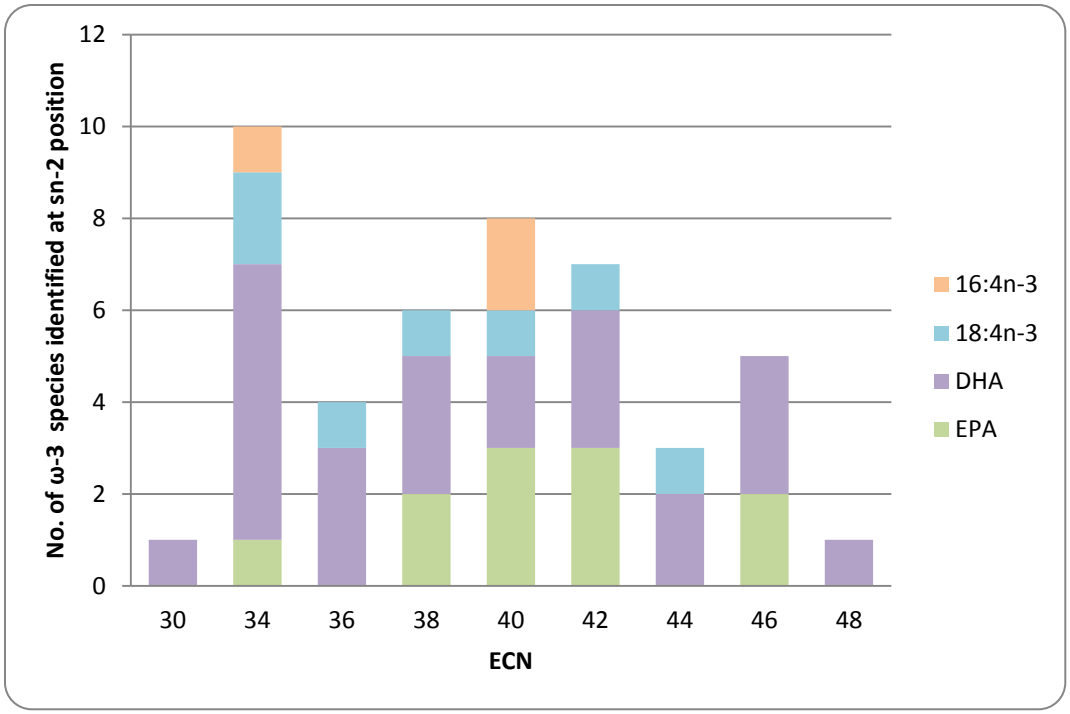

Figure 3.3 Number of ω-3 PUFAs at *sn*-2 position versus equivalent carbon number (ECN) for cod liver oil

3.5.2.2 Discrimination of mammalian and fish oils by LC-MS^2

A total of nine samples consisting of three seal oil samples (A, B and C) and six fish samples (2 cod liver , 1 herring, 1 salmon, 1 sand eel and 1 blue whiting) were used for discrimination purposes based on their ω-3 PUFAs content at *sn*-2 position.

The data from the predicting algorithm were arranged in ascending order based on ECN and the number of PUFAs in *sn-2* position were counted, tabulated and presented as histogram as shown in Fig 3.4. Unfortunately, the *sn*-2 approach does not give a clear separation for the analyzed mammalian and fish oils.

The number of EPA in blue whiting, cod liver, salmon and sand eel were higher when compared to the number DHA in the *sn*-2 position (Fig 3.4). The opposite trend was observed in seal oil (DHA > EPA in the *sn*-2). The observed difference could be important for customers who want to use marine oils for therapeutic purpose since, there is a markedly difference between EPA and DHA in their therapeutic action in the body. EPA is generally

used for its anti-inflammatory properties while DHA is mainly used for its neuroprotective and cardioprotective actions in the body [93].

Cod liver, herring and blue whiting exhibited a low number of PUFAs at the *sn*-2 position, even in herring the number of PUFAs was lower than that of seal oil, which contradicts earlier research reports where it is established that the number of PUFAs at *sn*-2 are higher in fish oils when compare to mammal oils [85]. It was puzzling that the stereospecific positioning of PUFAs in seal oil and cod liver oil presented in Figs 3.2 and 3.3 respectively are in accordance with published reports. However, the results of the present section (Fig 3.4) are in frank contradiction with the published reports. Based on the experimental results, it is possible to conclude that the seal and cod liver oils used in GC and Section 3.5.2.1 were genuine oils kindly donated by the oil providers while those used in this section were commercially available and purchased in the pharmacy.

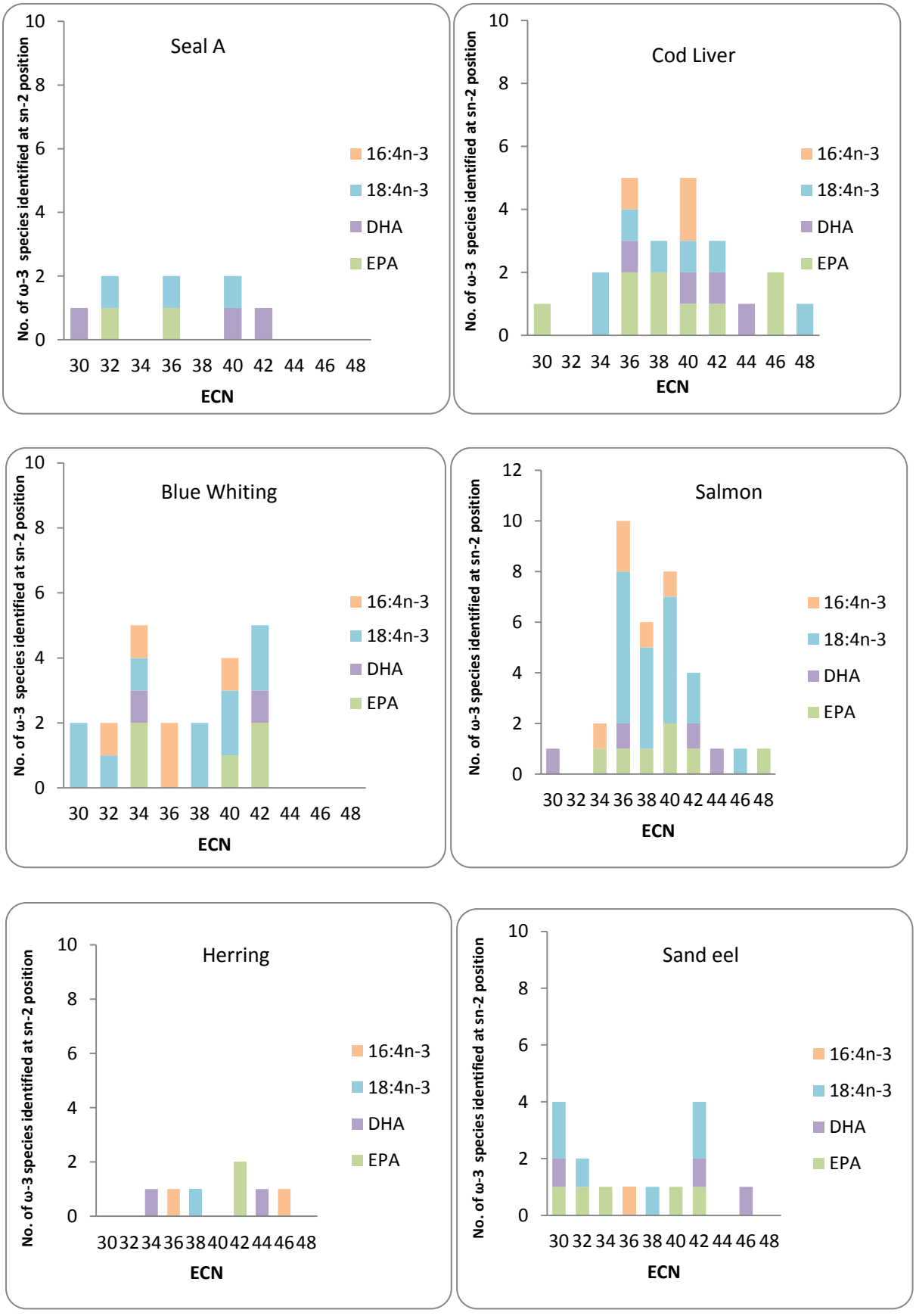

Figure 3.4 Histogram of different marine oils.

In general, Fig 3.4 revealed that there are few numbers of ω-3 PUFAs at *sn*-2 position in fish oils, which contradict the general literature. Based on these unexpected results, an important questions arise: Is it possible that the natural position of PUFAs on TAG molecules is affected by some lurking factors?

A literature review revealed that factors such as species, production method (wild or farmed), geographical origin and process history (natural fish oil or concentrated) can have an impact on the positioning of PUFAs [43, 94]. The few numbers of ω-3 at *sn*-2 positions in this section could be ascribed to the production history (see Section 3.5.2.3).

3.5.2.3 Genuine and processed fish oil

It is common knowledge that fish oil contains ω-3 PUFAs located at the *sn*-2 position of TAG molecules. However, some contradictory results were found in the previous sections. For example, the results in Section 3.5.2.1 indicated that the analyzed cod liver oil was genuine oil exhibiting a high number ω-3 PUFAs at position *sn*-2, which is approximately four times more than seal oil. Conversely, the results in Section 3.5.2.2 indicated that the analyzed cod liver oil was a processed oil due to its low content of ω-3 PUFAs at position *sn*-2, which is only approximately twice of seal oil.

The results (Figs 3.5 and 3.6) have clearly shown the difference between the numbers of selected ω-3 PUFA at *sn*-2 position of the genuine and processed oils. The genuine cod liver oil contains 70% EPA and 73% DHA at position *sn*-2, while in processed cod liver contains only 18.4% of EPA and 16% DHA at position *sn*-2. The observed difference between the two oils seems to indicate that the processing method applied to concentrate PUFAs in the oils may result in migration of fatty acids (e.g. PUFA) from *sn*-2 to the terminal position (*sn*-1 and *sn*-3) in TAG molecules [93].

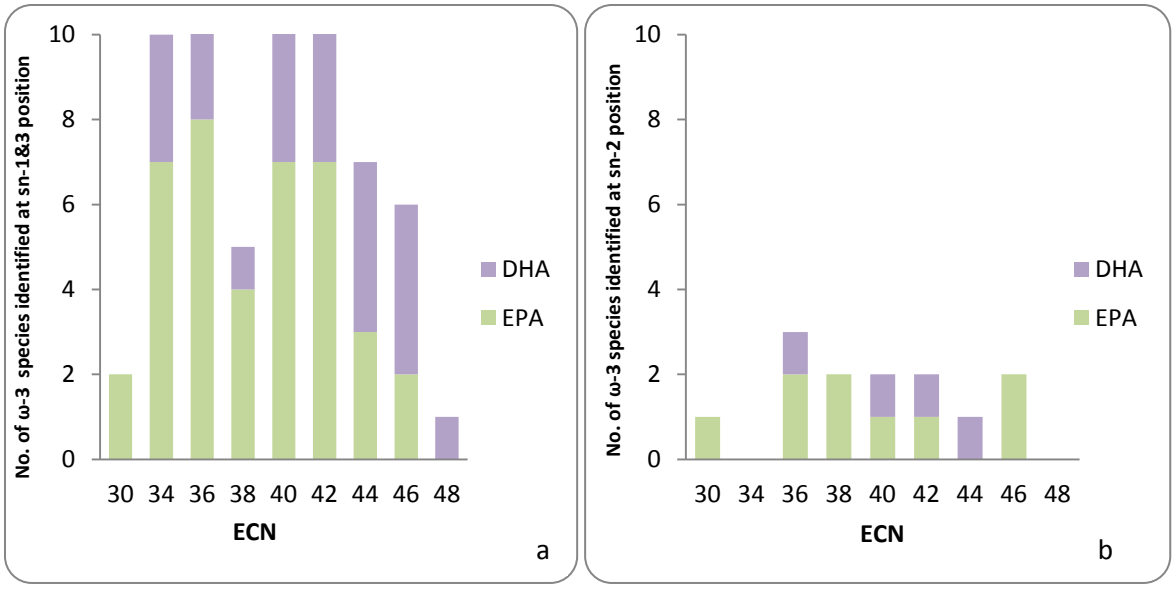

Figure 3.5: Histogram of processed cod liver oil a) at *sn*-1/ *sn*-3 and b) *sn*-2 positions of TAG structures

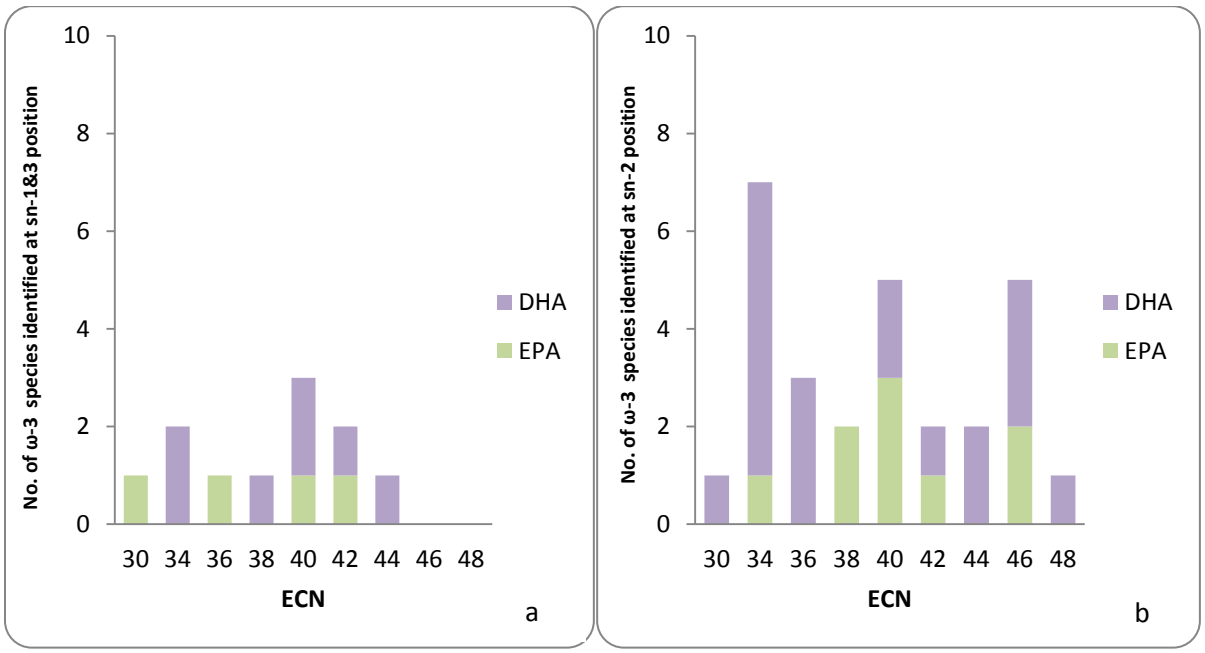

Figure 3.6 Histogram of genuine cod liver oil a) at *sn*-1/*sn* -3 and b) *sn*-2 positions of TAG structures

3.6 Conclusions

The primary goal of this section was to discriminate oils based on the number of ω-3 PUFAs at *sn*-2 position of TAG structures. The results revealed that any discrimination based on the *sn*-2 position could be a reasonable alternative for discriminating genuine from processed fish oils and also for distinguishing genuine fish and marine mammalian oils. It is important to emphasize that the bioavailability and absorption of PUFAs in the intestine could be altered by the position of individual fatty acids on the TAG backbone [20]. Consequently, any research aiming at testing the beneficial properties of marine oils should take into account whether or not their ω-3 PUFAs are located in the middle or terminal positions of TAG structures. The knowledge about the specific position of different ω-3 PUFAs on the backbone of TAGs could prevent misleading product labeling.

4. A new approach to discriminate marine oils by using the *sn*-1, *sn*-2 and *sn*-3 stereo specific positions of omega-3 polyunsaturated fatty acids on the backbone of triacylglycerol molecules.

4.1 Background

Dietary oils contribute a good share to the human diet and knowledge of their quality is of importance to prevent any health hazard. The consumer of the 21st century have developed an awareness of the quality of food products in their diet and the impact of these products on their health which in turn has led to an escalating consumer demand for ω-3 fatty acids rich oils, functional food, dietary supplements and pharmaceuticals.

The quality of marine oils may vary significantly according to the origins and the manufacture of the raw materials, and it is therefore essential to establish reliable analytical methods in order to carry out the quality assessment and authentication work on these kinds of products. The importance of developing techniques aiming at detecting adulteration of marine oils has been emphasised more than 100 years ago [44]. However, it has been much neglected compared to the discrimination of plant oils. Nowadays, dietary oils authenticity has become a focal point for the food industry, the policy makers, the international trade, research institutes and the consumers.The need for quality assessment of marine oils is mainly attributed to their content of ω-3 PUFAs and their beneficial effects on the heart, brain, joints, skin and even pregnancy. Several studies have evaluated the multiple ways that ω-3 PUFAs promote cardiovascular health, in addition to the healthy functioning of many other biological activities [6-9].

Marine oils are rich in TAGs (> 98 %), which comprises of fatty acids esterified to three stereospecific positions on the glycerol backbone. The positions of these fatty acids are numbered relative to their stereospecific positioning patterns as described in Section 1.1.6. By using proper analytical instrumentation for TAG analysis such as LC-MS and NMR, the positioning patterns of TAG derived from the intact TAG can be determined. The TAG patterns provide information not only on fatty acids composition but also on the stereospecificity of fatty acids on TAG molecules. Compared to the composition analysis of

simple fatty acids, TAG patterns usually carry more information and could be used for determining the quality and authentication purposes.

A literature review (Table 2) has revealed that the development of different instrumental methods for marine oil authenticity is mainly based on composition and stereospecific positioning of fatty acids. Traditionally, the positional analysis of TAGs is performed by means of laborious and time-consuming chromatographic and enzymatic methods [31, 89-91]. Recently, various spectroscopic techniques such as ^{13}C NMR [34-38], ESI MS [39], and APCI-MS [40] methods have been implemented. NMR is the most frequently employed technique for authentication of marine oils based on stereospecific positioning as indicated in the Table 2. Authentication based on the steriospecific positioning *sn*-2 of ω-3 PUFAs as demonstrated in the previous chapter was acceptable for distinguishing between genuine and processed fish oils. In the present section a new strategy is explored and proposed to distinguish different marine oils. The new strategy is also implemented in the authentication of intentionally adulterated oils.

4.2 Objectives

- To establish a new normalization strategy (NNS) based on ECN and the ratio of outer/inner ω-3 PUFAs on TAG structures from marine oils.
- To assess the NNS on intentionally adulterated oils of nutritional importance.

4.3 Experimental

4.3.1 Reagents and samples

Methanol (HPLC grade, ≥99.9%), acetonitrile (LC grade, ≥99.8%), ammonium acetate (mass spectrometry grade, 99%) and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hexane**,** LC grade was from Merck (Darmstadt, Germany). Isopropanol used for HPLC was from Kemetyl (Norway). De-ionized water was used throughout the experiment and purified in a Milli-Q system (Millipore, Milford, USA). Cod liver oils were from Peter Möller, Lysaker and Axellus AS,Oslo, Norway. The salmon oil was from Havnegater, Sortland, Norway. Two different batch harp seal oil (*Phagophilus groenlandicus*) were from

Rieber Skinn A/S, Bergen, Norway and the other seal oil was from JFM Sunile A/S, Os, Norway. The herring, blue whiting and sand eel were kindly donated by Veronika Sele from NIFES.

4.3.2 Sample preparation

An aliquote of 2 mL of each oil was taken and submitted to the optimized LLE method as described in Chapter 2. The final hexane fraction was dried under stream of nitrogen at room temperature. The dried residues of samples were dissolved into 0.5 mL of acetonitrile: acetone (2:1, v/v) and vortex-mixed for 30s. Every sample was prepared in duplicate and the final products were then individually sent to LC-ESI-MS^2 analysis.

4.3.3 LC ion-trap mass spectrometry instrumentation

The TAG analysis were carried out by using an Agilent 1100 series LC/MSD trap, SL model with an electrospray interface, a quaternary pump, degasser, autosampler, thermostatted column compartment, variable-wavelength UV detector and 10 µl injection volume. The Zorbax Eclipse-C8 RP 150 × 4.6 mm, 5 µm (Agilent Technologies, Palo Alto, CA) was kept in the column compartment at 40 °C and the solvent system in gradient mode consisted of acetonitrile (A), acetone (B) and 10 mM isopropanol:ammonium acetate 90:10 v/v (C) at a flow rate of 0.2 ml/min and UV detection at 254 nm. The following gradient programs was used: at initial 5 min condition 90 % C and 10 % B that was ramped in 5 min to 65 % C,5 % B Plus 30% of A and returned to the initial condition in 15 min and subsequently ramped in 5 min to 65 % C,5 % B Plus 30% of A and returned to the initial condition in 30 min where it was held for 30 min. Nitrogen was used as nebulizing (50 psi) and drying gas (8 L/min) at 350 °C. The ESI source was operated in positive ion mode and the ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option. Data acquisition and processing were controlled by MSD trap control version 5.2 from Agilent.

4.3.4 LC-ESI- MS^2 Analysis data

All the TIC+MS^2 data was exported to netCDF file by Data Analysis for LC/MSD Trap Version 3.3 (Bruker Daltonik GmbH Inc., Billerica, MA, USA). The netCDF file was then

exported to a Matlab file and submitted to the developed automated triacylglycerol prediction algorithm for identification of TAG species in oil samples. The resulting data was transferred to and saved in Excel format.

4.3.5 New Normalization Strategy

It was demonstrated in previous section that the discrimination analysis of fish and marine mammal oils based on the number of ω-3 PUFAs at position *sn*-2 is a reliable approach for genuine oils where the position of ω-3 PUFAs is preserved. However, for oils that have been subjected to chemical, thermal, enzymatic and deodorizing processes; the discrimination analysis based on *sn*-2 position is no longer valid due to the migration of the ω-3 PUFAs from the inner to the outer position of TAG structures (Fig 4.1).

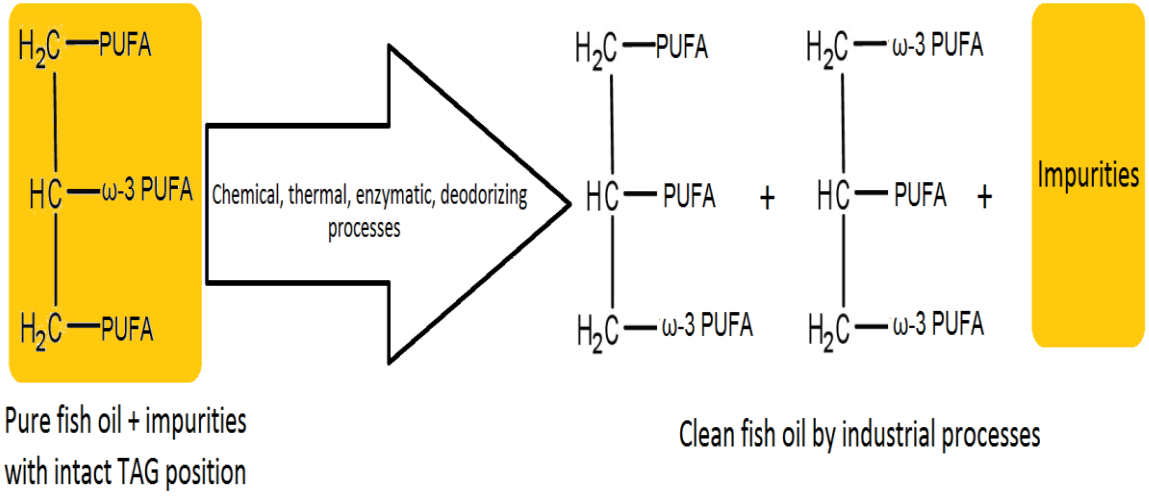

Figure 4.1 Schematic representation of the migration of ω-3 PUFA from inner to outer TAG position after processing of pure fish oil

The present section aims at proposing a new normalization strategy (NNS) that allows discriminating oils regardless of their purity or migration of ω-3 PUFAs from inner to outer positions of TAG structures. The proposed NNS which consists of four steps is explained by using a hypothetical oil named HyO:

I. The identified TAG species in HyO by using LC-MS^2 and the TAG prediction algorithm (described in Chapter 3, subsection 3.5.2) are arranged in increasing number of ECN.

ECN	***sn*-1**	***sn*-2**	***sn*-3**
30	16:1n	16:4n-3	EPA
30	18:4n-3	EPA	EPA
34	EPA	18:4n-3	EPA
34	16:1n	16:4n-3	16:1n
34	18:4n-3	14:00	18:4n-3
36	18:4n-3	16:1n	18:4n-3
36	EPA	DHA	EPA
38	16:1n	16:1n	18:4n-3
38	16:1n	18:4n-3	16:1n
40	16:4n-3	16.00	18:4n-3
40	EPA	DHA	EPA
42	16.00	EPA	16.00
42	16.00	18:1n	18:4n-3
42	18:1n	16.00	EPA
44	18:1n	DHA	18:1n
44	EPA	18:4n-3	EPA
46	20:1n	20:1n	DHA
46	18:1n	EPA	22:1n
48	EPA	20:1n	22:1n
48	18:1n	DHA	18:1n

II. The total number of ω-3 PUFAs at the terminal positions (*sn*-1 and *sn*-3 added together) and at *sn*-2 are counted for each ECN. For example, the total number of ω-3 PUFAs at ECN 30 and position *sn*-2 are 2 (16:4n-3 + EPA = 2) in the above table while for the same ECN the summation of ω-3 PUFAs at positions *sn*-1+*sn*-3 is 3 (18:4n-3 + EPA + EPA = 3)

ECN	*sn*-1 + *sn*-3	*sn*-2
30	3	2
34	4	2
36	4	1
38	1	1
40	4	1
42	2	1
44	2	2
46	1	1
48	1	1

III. At every ECN, the total counted ω-3 PUFAs at terminal positions (*sn*-1 + *sn*-3) are divided by the corresponding total counted ω-3 PUFAs at *sn*-2 position and the ratio outer/inner determined as indicated in the below table

ECN	(*sn*-1 + *sn*-3)/ *sn*-2	Ratio outer/inner (O/I)
30	3/2	1.5
34	4/2	2
36	4/1	4
38	1/1	1
40	4/1	4
42	2/1	2
44	2/2	1
46	1/1	1
48	1/1	1

IV. The highest ratio outer/inner is used to normalized the previous ratios. For example, the maximum ratio 4 corresponds to ECNs 36 and 40 and it is subsequently used for normalizing the rest of the outer/inner ratios as follows:

ECN	Ratio outer/inner (O/I)	(O/I)/4	Normalized values (NV)
30	1.5	1.5/4	0.4
34	2	2/4	0.5
36	4	4/4	1.0
38	1	1/4	0.3
40	4	4/4	1.0
42	2	2/4	0.5
44	1	1/4	0.3
46	1	1/4	0.3
48	1	1/3	0.3

The four steps procedure (I-IV) is applied to every analyzed oil. The normalized values (NV) for the various samples are arranged as NV rows and ECN columns (NV×ECN).

4.3.6 Discrimination analysis

To examine the discrimination between mammalian and fish oils, six different pure oils (seal, salmon, cod liver, sand eel, blue whiting, herring) and a mixture of oils (containing blue whiting, herring, sand eel and Norway pout fish oil) were used. For discriminate genuine and adulterated marine oils, two different kinds of oils (cod liver and blue whiting) were used to adulterate pure seal oil. The adulterants were evaluated at three different concentration levels (25, 50 and 75 %). Duplicates samples were prepared for pure seal, cod liver and all level of seal adulterated cod liver oils. The discrimination of the various samples was performed by means of principal component analysis (PCA) using their total ion current (TIC) chromatograms.

All the TIC+MS^2 data was exported to netCDF file by Data Analysis for LC/MSD Trap Version 3.3 (Bruker Daltonik GmbH Inc., Billerica, MA, USA). The netCDF file was then exported to a Matlab file and submitted to the developed automated triacylglycerol prediction algorithm for identification of TAG species in oil samples. The results were arranged in increasing number of ECN and all ω-3 PUFAs at *sn*-2 positions were counted, tabulated and treated by the above normalized strategy (Section 4.3.5).The matrix of normalized values and

ECN (NV x ECN) is subjected to PCA and two-dimensional PCA score plots were used to visualize discrimination between various marine oils. The PCA score plot of the normalized value profiles from the various oils was computed with the software package Statistica version 12 (Statsoft. Inc.1984-2014).

4.4 Results and discussion

4.4.1 Discrimination study of different kinds of marine oils based on NNS

The marine oils were analyzed in duplicate, submitted to the automated algorithm and normalized as stated in Section 4.3.5. The normalized values were arranged in matrix consisting of 20 rows representing the NV of the different analyzed oils along with their replicates and 10 columns representing the equivalent carbon number (ECN).

PCA was applied to check whether or not the proposed NNS can assist in discriminating different kind of marine oils regardless of their production process. PCA is frequently employed for the purpose of generating a reduced set of variables that account for the most of the variability in the original data, and the first principal component score (PC1) contains the most representative information in the data set.

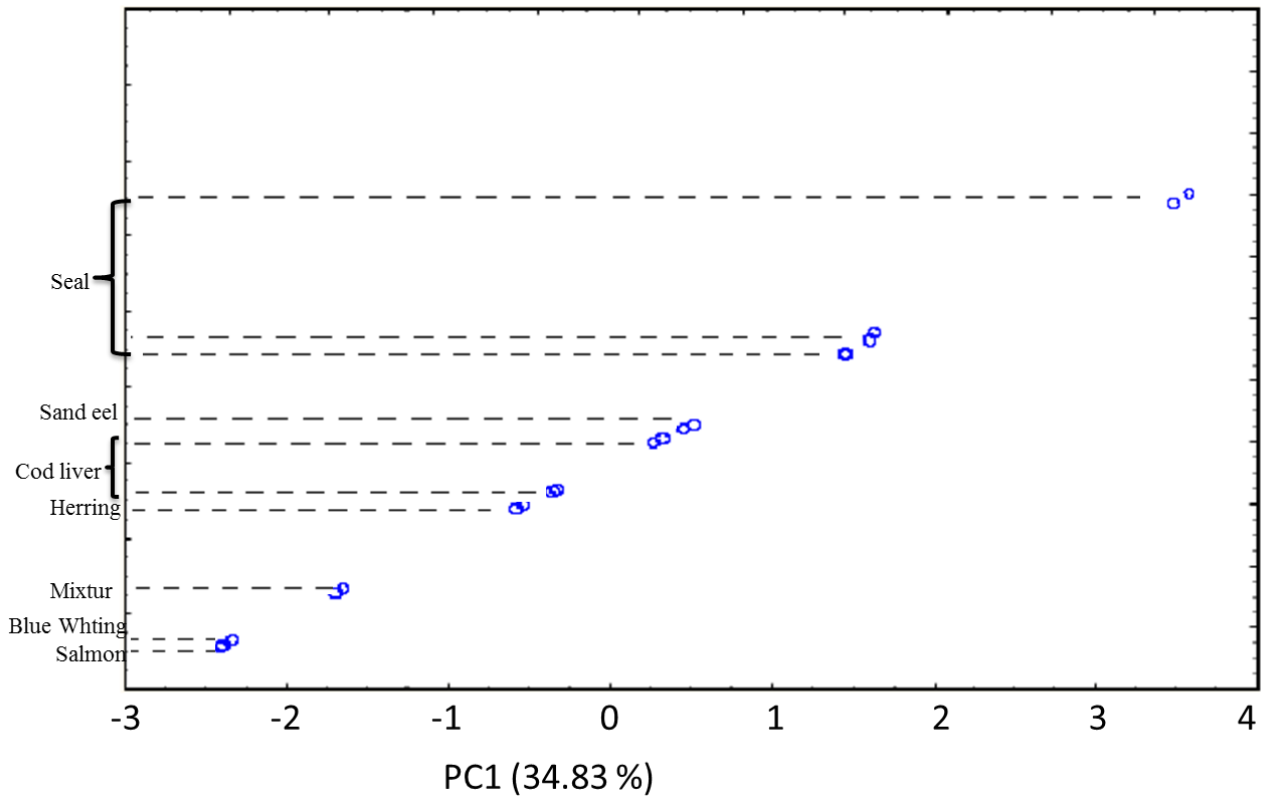

Figure 4.2 PC1 score plot for the different marine oils analyzed

As shown in Fig 4.2, PC1 explains 34.83 % of the total variation and also discriminate the oils according to their nature. The mammalian and fish oils are clearly separated (Fig 4.2) in which seal oils clustered themselves in one end of PC1. In addition, PC1 discriminate between seal oils samples from different manufacturer (i.e. the closest two seal oils from the same manufacturer). At the middle of the PC1 axis the two cod liver oils are grouped together with herring and sand eel oils, this may be attributed to their similarity in feeding behavior and location under water [95,96], which in turn affect the TAG composition of the fish.[43].

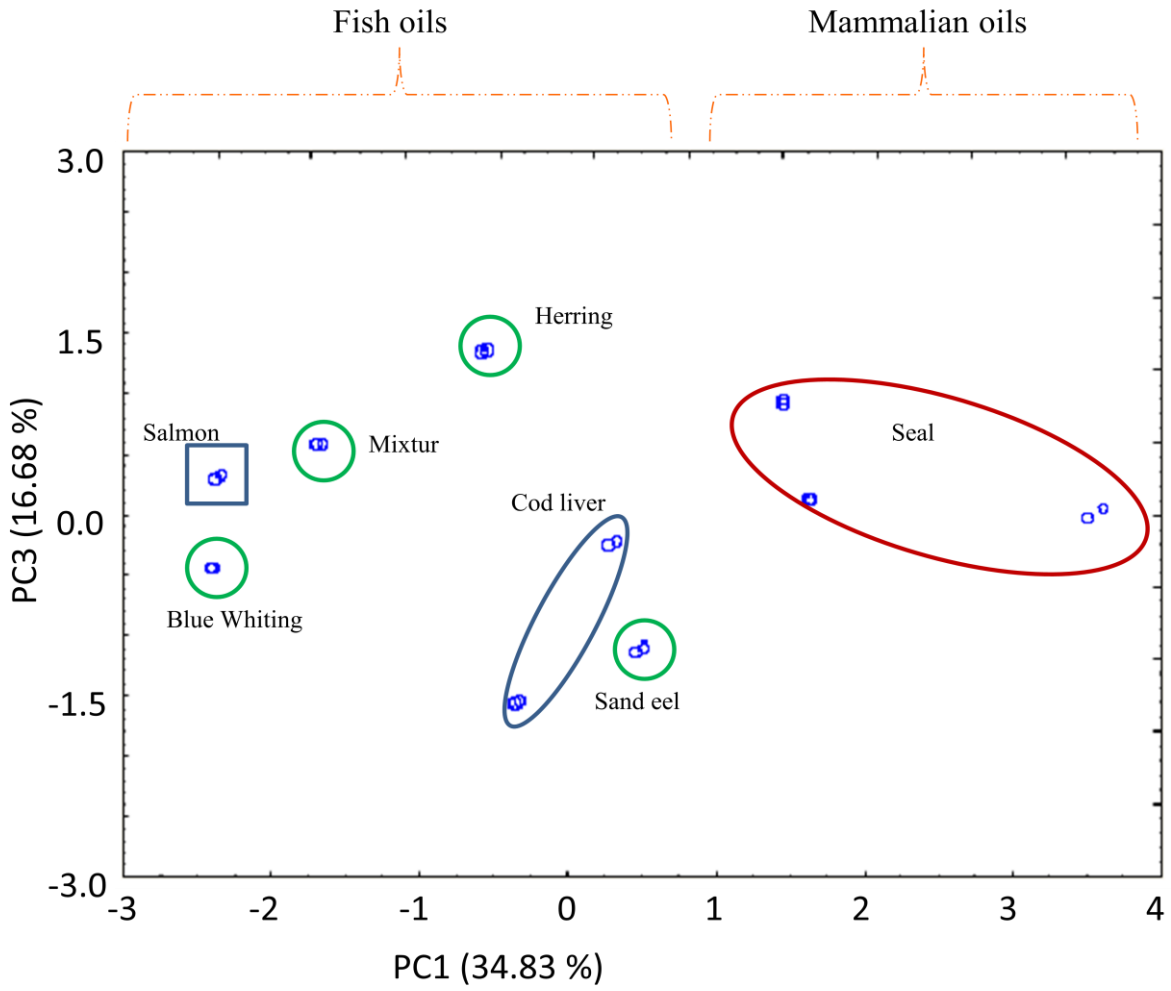

Figure 4.3 PC1 and PC3score plot for the different analyzed marine oils

The PC1 and PC3 score plot explains 51.51 % of the total variance of which 34.83 % and 16.68 % of the variation is accounted by the former and latter PC respectively (Fig 4.3). The PCA plot (Fig 4.3) shows the similarities and differences of the samples, wherein similar samples tend to form clusters and dissimilar samples are separated away. It is clear from Fig 4.3 that the oil samples are classified into three distinct clusters, which are seal oil (three seal oil samples), two cod liver and sand eel oils, and the other fish oils (salmon, blue whiting and herring oil samples). PC1 shows clear separation of seal (mammalian) oils from fish oils. While seal oils are located on the positive side of PC1, the salmon, blue whiting and herring were on the negative side of PC1. It also observed that the two different batches of seal oils from the same manufacturer and the two cod livers were discriminated by PC3, which indicate some differences within them. Sand eel oil grouped with the two cod liver oils, this

could be the result of sharing the same habitat and also it could reflect the dietary habits of cod fish which in its adulthood start hunting sand eel [43, 95]. On the other hand the mixed sample, which contains blue whiting, herring, sand eel and Norway pout, is located in between of its original component oils. It is clear from Fig 4.3 that the mixture is between blue whiting and herring oils but far from sand eel, possibly due to the effect of the Norway pout oil, which was not analyzed in this study.

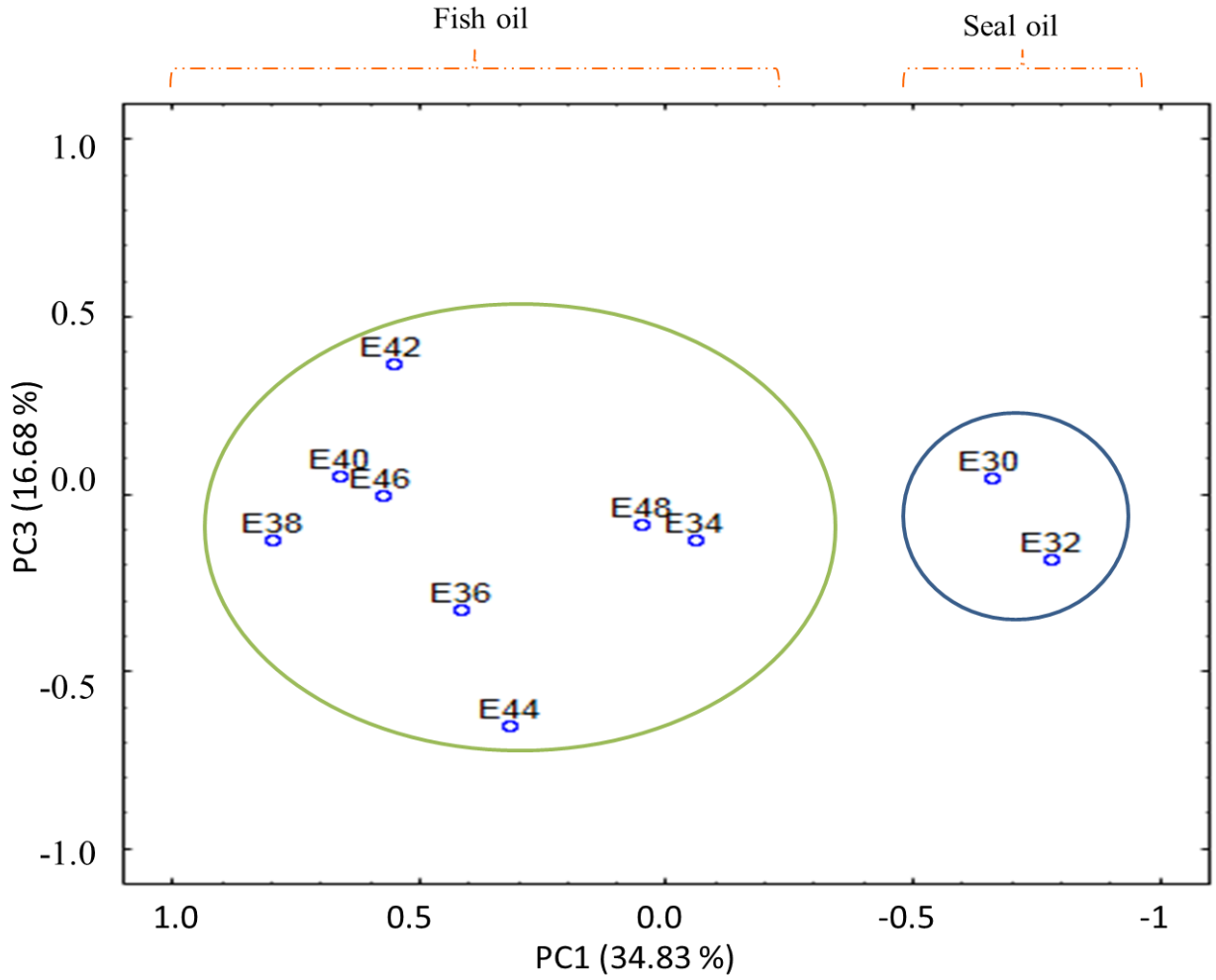

Figure 4.4 PC1 and PC3 loading plot and its relation with the score depicted in Fig 4.3

The loading plot of PC1 and PC3 representing the relationship between various equivalent carbon numbers is showed in Fig 4.4. The superimposition of the two (fish and mammalian) clusters from score plot (Fig 4.3) on the loading plot (Fig 4.4) demonstrates that in general there is an opposite correlation between ECN 30 and 32 and the rest of the ECN (the ECN are denoted by the letter E in Fig 4.4). The loading values of E30 and E32 are negative while the

rest E36-E48 exhibit positive loading values. The clustering of loadings in positive and negative sides of PC1 is responsible for the discrimination between seal oil and fish oils. The above observation is in accordance with the results of Chapter 3 where it was demonstrated (Figs 3.2-3.6) that seal contains ω-3 in ECN 32 whereas it is absent in the other fish oils except sand eel and blue whiting. Similarly, ω-3 is present in ECN 34 of fish oils but absent on seal oil. The loading plot (Fig 4.4) seems to indicate that ECN 32 and ECN 34 could be a good indicator for discriminating between seal and fish oils. However, more research in this respect would be advisable.

4.4.2 Discrimination study of adulterated marine oils based on NNS

The converted data of the TIC chromatograms were studied by PCA to evaluate if the number of ω-3 PUFAs identified on the backbone of TAG enables the discrimination of pure from adulterated oils. The adulterants were evaluated in duplicate by adding to pure seal oil different levels of cod liver oil adulterant (0, 25, 50, 75 and 100 %). The normalized values were arranged in matrix consisting of 10 rows representing the different analyzed oils (pure and adulterated) with their replicates and 10 columns represent the individual ECN and submitted to PCA. The first principal component score plot (Fig 4.5) explains 60.99 % of the total variation and the information retained by this component provides a clear differentiation between genuine and adulterated oils.

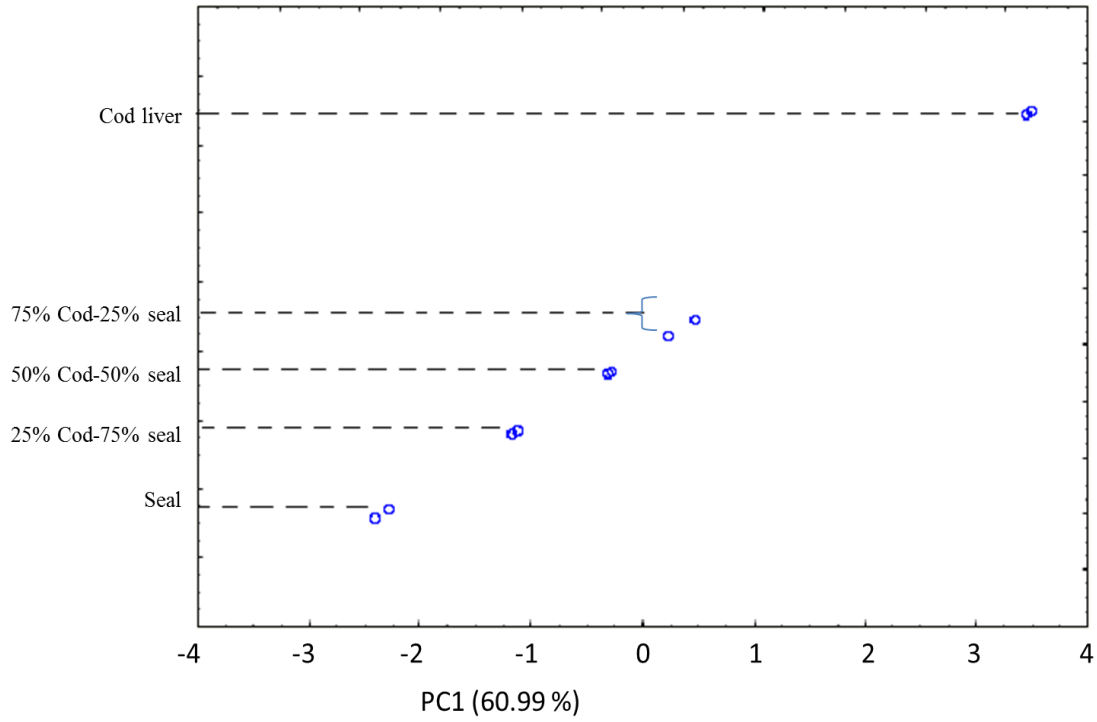

Figure 4.5 PC1 score plot for pure seal (or cod liver) oil and four level of adulterant

The score of the pure cod liver oil samples were clustered together and located in one end of the PC1 axis while pure seal oil in turn clustered together and located at the opposite end of the axis. On the other hand the three level adulterated oils (seal or cod liver oil) in this study (25, 50 and 75 %), were located at the middle of the axis. The pure seal oil clearly separated from adulterated with cod liver at the three levels of impurities and the relative position of adulterated oils are based on the percentage of adulterant. For instance the sample, which contain 25 % of seal and 75 % of cod liver oil by volume (denoted by 75% cod - 25% seal) were found closer to pure cod liver oil compared to other adulterated samples, hence it contain three times more cod liver than seal oil and the same is applicable to the opposite ratio of adulteration. Pure cod liver were relatively far from the rest of the oils this may be attributed to the presence of high number of ω-3 PUFAs in *sn*-2 position and significant amount on *sn*-1 and 3 position in cod liver compared to seal, conversely seal contain very few ω-3 in *sn*-2 and very high on *sn*-1 and 3 positions, which is responsible for the high normalized value of seal oil. The adulterated oils are close to each other and pure seal oil, these could be the addition of cod liver oil which increases the ω-3 in the terminal position than in *sn*-2 position. The detection of cod liver oil as adulterant of seal oil may be difficult due to their strong resemblance [87,97-98]. However, this problem can be solved by

comparing pure and adulterated cod liver sample, since high number of ω-3 PUFAs are located at the *sn*-2 position in pure cod liver oil, while for adulterated (25, 50 or 75 %) a significant number of ω-3 PUFAs not only at the *sn*-1 and 3 positions but also at the *sn*-2 positions which could indicated the presence of cod liver oil.

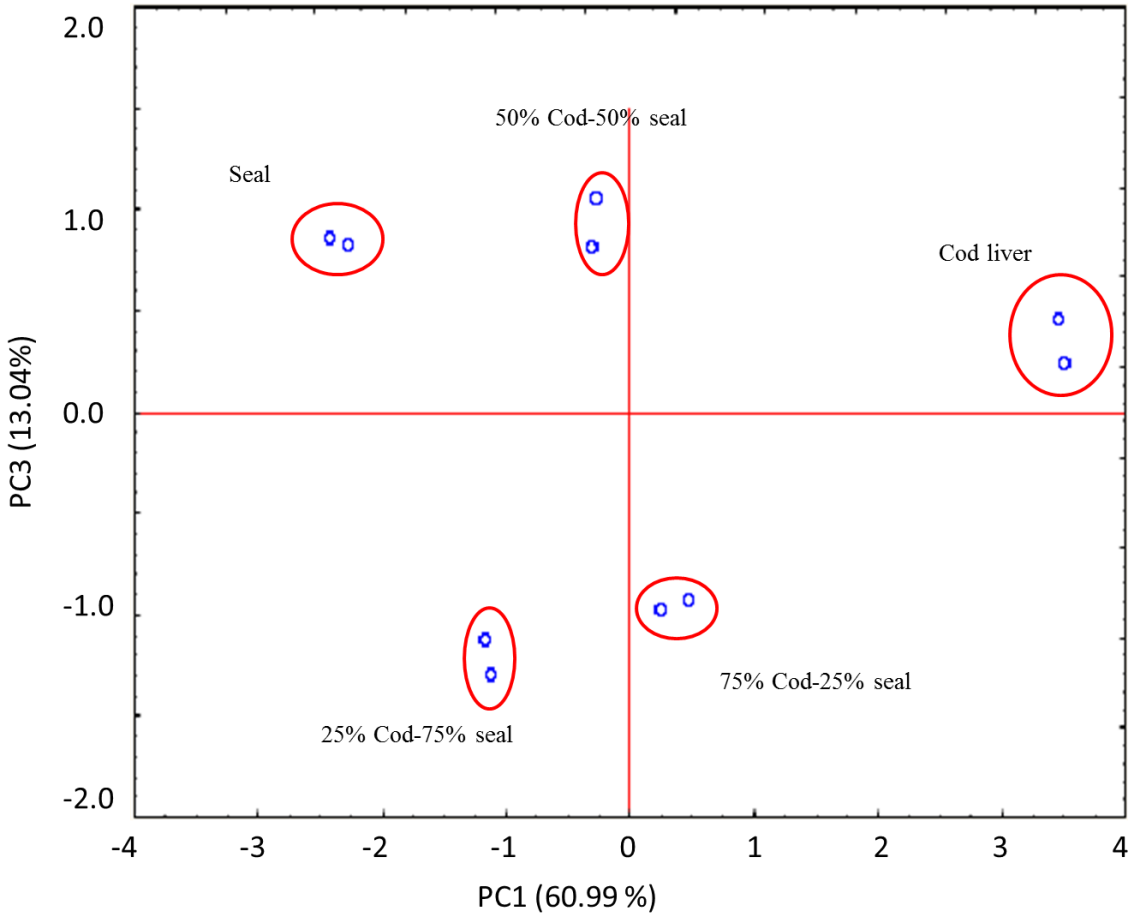

Figure 4.6 PC1 and PC3 score plot for pure seal (or cod liver) oil and four level of adulterant

PC1 (60.99%) and PC3 (13.04%) explained 74.03 %, of the variability of the samples, indicating that the extracted principal components could reflect excellently the majority of information of the original data. Pure cod liver and adulterated samples with high percentage of cod liver are located on the positive side of PC1 (Fig 4.6) while pure seal and adulterated sample with low percentage of cod liver are negatively located on PC1. The upper part of the score, which is in the positive side of PC3 contain the pure samples and adulterated with equal volume of the two marine oils, while the bottom part of the score in the negative side of PC3

are the two adulterated oils with uneven percentage of the oils. This means PC3 could also provide some information about the degree of adulteration.

Furthermore, to verify the discrimination capability of the proposed LLE method and the NNS approach in oils of national commercial importance, pure seal and pure salmon were tainted with blue whiting oil (0, 50 and 100 %). The score plot of the first principal components (Fig 4.7) explained 48.72% of the total variation and discriminated pure and adulterated oils. The PC1 clearly shows the adulterated oils are located in between the two pure oils, for instance, the sample seal: blue whiting (1:1) is located between pure seal (negative PC1) and pure blue whiting (positive PC1). The same behavior is observed for salmon: blue whiting (1:1).

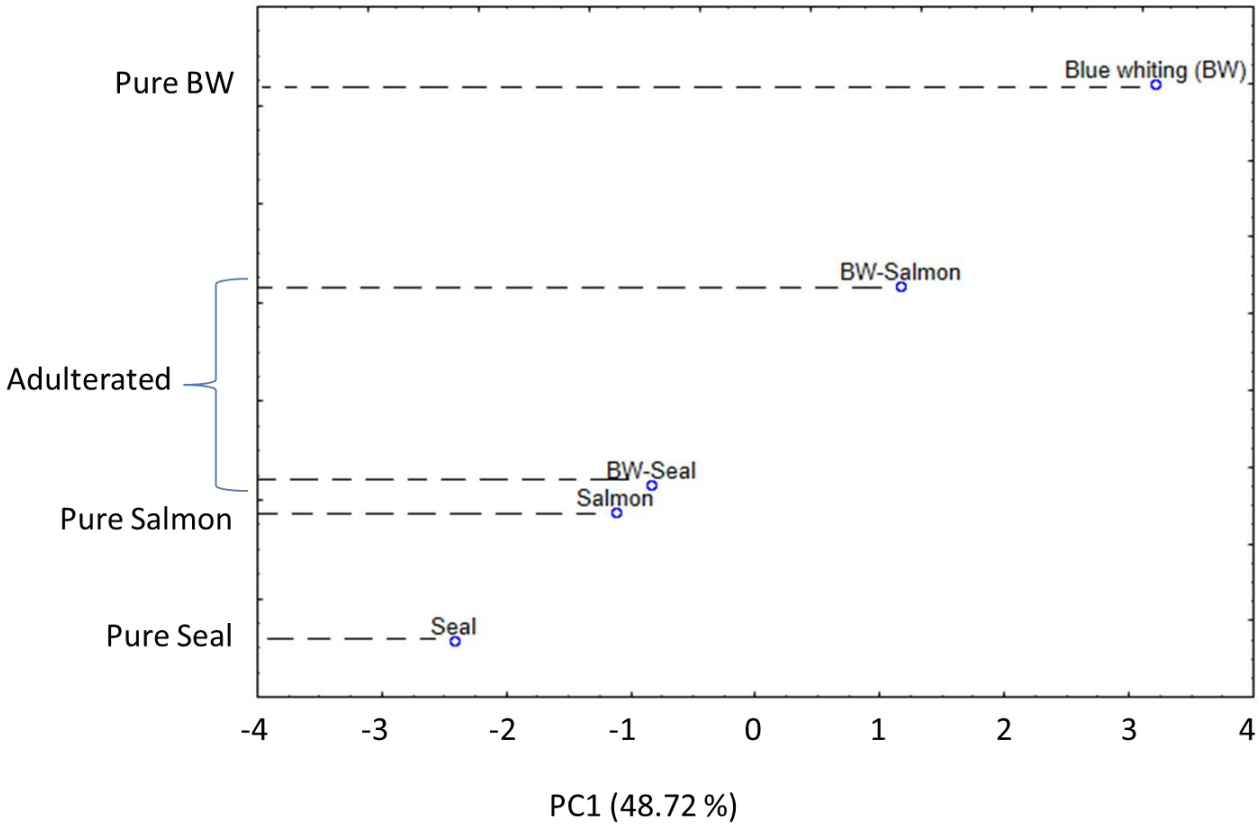

Figure 4.7 PC1 score plot of pure seal and pure salmon oils tainted with 0, 50 and 100 % blue whiting oil

4.5 Conclusions

It would be interesting to include a larger number of mammalian and fish oils to determine whether or not the ECN (E32 and E34) could be a good parameter for discriminating between mammalian and fish oils.

The new strategies proposed in the present research such as the LLE method and the NNS have demonstrated to be extremely valuable tools for testing the purity of nutritional marine oils, such as seal, cod liver and salmon. Consequently, they could have a positive impact nationally and internationally in the frame of the food industry and trade.

5. Concluding remarks

This thesis developed a simple approach for extracting lipids from nutritional marine oils and proposed two new strategies for discriminating different kinds of marine oils. In addition, the thesis shows for the first time that it is possible to extract quantitative data from the TAG prediction algorithm (developed by a former EMQAL student) for discrimination and authenticity purposes.

The developed LLE allows separating TAG and PL from krill oil samples by using two solvents (methanol and hexane) without the need of HPLC or HPTLC separations. For his reason, the proposed approach could be regarded as an important alternative especially for laboratories where the lack of chromatographic equipments is a limitation for performing analytical or preparative separations.

The first strategy to discriminate marine oils and based on the number of ω-3 PUFAs located at the *sn-2* position of TAG molecules is a powerful tool for discriminating not only fish from marine mammalian oils, but also to determine whether or not the *sn-2* position of ω-3 PUFAs in fish oil has been altered during the refining process.

The second approach to discriminate nutritional marine oils is based on the ratio of outer/inner ω-3 PUFAs on TAG structures by using a new normalization strategy (NNS) which has demonstrated a great potential for authenticating nutritional oils.

The most important feature of the present thesis is that the various developed aspects (extraction and discrimination) can be implemented in laboratories where the lack of instrumental equipments and specialized software can represent a serious constraint for the intended analysis. For example, nowadays, NIFES can isolate TAG (without the need of HPLC or HPTLC), determine the fatty acid positioning (using LCMS and the TAG prediction algorithm) and perform a PCA analysis (without the need of CODA for preprocessing the full LCMS profiles) with data from the TAG prediction algorithm and the NNS approach.

Future studies on discrimination and authentication of nutritional oils should consider:

- ✓ The inclusion of a higher number of genuine and processed fish oil samples to validate the strategy based on the number of ω-3 PUFAs at the *sn-2* position.

- ✓ The inclusion of a higher number of marine mammals for discrimination studies based on the outer/inner ω-3 PUFAs position and the NNS methodologies

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